ANTIMICROBIAL ACTIVITY OF *PHYLLANTHUS EMBLCA* FRUIT EXTRACT AND ITS COSMETIC APPLICATION

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for the Degree of Master of Science Program in Pharmaceutical Technology
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ฤทธิ์ต้านจุลินทรีย์ของสารสกัดผลมะขามป้อมและการประยุกต์ด้านเครื่องสำอาง

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ลัดดาวัลย์ เชื้อเจ็ดตน: ฤทธิ์ต้านจุลินทรีย์ของสารสกัดผลมะขามป้อมและการ ประยุกต์ด้านเครื่องสำอาง (ANTIMICROBIAL ACTIVITY OF *PHYLLANTHUS EMBLICA* FRUIT EXTRACT AND ITS COSMETIC APPLICATION) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วลัยศิริ ม่วงศิริ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร.พรเพ็ญ วีระวัฒกานนท์, 123 หน้า.

สารสกัดจากผลมะขามป้อมมีฤทธิ์ทางชีววิทยาหลากหลาย ได้แก่ ฤทธิ์ต้านจุลินทรีย์ และฤทธิ์ต้านการทำงานเอนไซม์ไทโรซิเนส การนำสารสกัดผลมะขามป้อมมาผสมในเครื่องสำอางจึงเป็นการเพิ่มมูลค่าทางเศรษฐกิจและเพิ่มทางเลือกในการรักษาอาการ เช่น สิวให้กับผู้บริโภค ในการวิจัยนี้ได้นำสารสกัดผลมะขามป้อมที่มีขายและสารสกัดผลมะขามป้อมที่ทำการสกัดเอง (ESDE) นำมาสกัดด้วยด้วยอะซีโตน เมทานอล และเอธิลอะซีเตท กากที่เหลือจากการสกัดด้วยเอธิลอะซีเตทถูกสกัดต่อด้วยอะซีโตนและเมทานอล สิ่งสกัดแต่ละชนิดจะถูกนำมาทดสอบลักษณะ ฤทธิ์ต้านจุลินทรีย์และหาปริมาณของสารประกอบฟีนอลิกด้วย HPLC หรือ Folin-Ciocalteu reagent นำสกัดมะขามป้อมมาเตรียมเป็นเจลโดยใช้ sodium metabisulfite, sodium benzoate และ citrate buffer pH 3.0 นำไปประเมินความคงตัวทางกายภาพและทางเคมี โดยเก็บเจลที่อุณหภูมิ 30 $\pm$ 2 ^{o}C ความชื้นสัมพัทธ์ 75 $\pm$ 5 % เป็นเวลา 1 เดือน และทดสอบฤทธิ์ต้านสิวกับอาสาสมัครจำนวน 25 คน

จากการทดสอบค่าทางเคมีพบว่า ESDE และสิ่งสกัดพบว่ามีปริมาณสารฟีนอลิค 15.77-29.92% w/w ซึ่งมากกว่าสารสกัดผลมะขามป้อมที่มีขายและสิ่งสกัด ESDE มีค่า MIC ต่อเชื้อ *S. aureus* ATCC 25923 และ *S. aureus* อีก 29 ไอโซเลท เท่ากับ 10 mg/mL ส่วนสิ่งสกัดอื่นๆ ไม่สามารถนำมาทดสอบหาค่า MIC ได้เนื่องจากความสามารถในการละลายน้ำต่ำ เมื่อวิเคราะห์ข้อมูลสารฟีนอลิคใน ESDE ที่ละลายด้วย citrate buffer ด้วยวิธี HPLC พบว่าพีคที่ 1 และแกลลิคแอซิดเพิ่มขึ้น ซึ่งอาจเกิดจากการสลายตัวของสารฟีนอลิค พีคที่ 2, 5 และ 6 ลดลง ส่วนพีคที่ 3 คงที่ พีคที่ 6 มีปริมาณลดลงอย่างชัดเจนภายใน 1 สัปดาห์ สอดคล้องกับการลดลงของความกว้างของโซนที่เกิดจากการยับยั้งเชื้อ *S. aureus* เจลที่มีส่วนผสมของสารสกัดผลมะขามป้อมมีสีเหลืองมัวไม่ทึบแสง เจลเปลี่ยนเป็นสีน้ำตาลภายใน 1 สัปดาห์และเป็นสีน้ำตาลเข้มภายใน 1 เดือน ค่า pH ตลอดระยะเวลา 1 เดือน เท่ากับ 3.24 $\pm$ 0.06 ค่าความหนืดตอนเริ่มต้นเท่ากับ 1238 $\pm$ 75.60 และ 1040 $\pm$ 21.79 cP ที่เวลา 1 เดือน ปริมาณแกลลิคแอซิดตอนเริ่มต้นเท่ากับ 0.06 และ 0.09 % w/w ที่เวลา 1 เดือน เมื่อวิเคราะห์ด้วย HPLC ปริมาณของพีคต่างๆ มีการเปลี่ยนแปลงสอดคล้องกับ ESDE ที่ละลายในซิเตรทบัฟเฟอร์ เจลที่มีส่วนผสมของสารสกัดผลมะขามป้อมถูกนำไปทดสอบฤทธิ์ต้านสิวเป็นเวลา 1 เดือนเปรียบเทียบกับกลุ่มควบคุมบวก 1% clindamycin gel พบว่าอาสาสมัครแต่ละคนมีปริมาณเม็ดสิวที่ลดลงโดยมีการเปลี่ยนแปลงอย่างชัดเจนระหว่างเวลาเริ่มต้นและที่สิ้นสุดสัปดาห์ที่ 4 ($p<0.05$) ที่สิ้นสุดของแต่ละสัปดาห์ พบว่าค่าเฉลี่ยร้อยละของเม็ดสิวที่ลดลงระหว่างอาสาสมัครสองกลุ่มไม่แตกต่าง ($p>0.05$)

ภาควิชา เทคโนโลยีเภสัชกรรมและเภสัชอุตสาหกรรม ลายมือชื่อนิสิต.....

สาขาวิชา เทคโนโลยีเภสัชกรรม ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา 2555 ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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KEYWORDS : Emblica extract/ Antimicrobial activity/ Anti-acne

LADDAWAN CHUAJEDTON: ANTIMICROBIAL ACTIVITY OF *PHYLLANTHUS EMBLICA* FRUIT EXTRACT AND ITS COSMETIC APPLICATION. ADVISOR: ASST. PROF. WALAISITI MUANGSIRI, Ph.D., CO-ADVISOR: ASST. PROF. PORNPEN WEERAWATGANONE, Ph.D., 123 pp.

Fruit extract from *Phyllanthus emblica* L., a native plant in Thailand, possesses several biological activities including antimicrobial and antityrosiness activities. Development of a cosmetic product containing emblica fruit extract is not only increase economic values of this native plant but also provides an alternative choice of natural anti-acne product for consumers. In this study, fruit extract powder from two different sources, a commercially available emblica extract and an in-house emblica extract (ESDE), was extracted with acetone, methanol and ethyl acetate. The marc from ethyl acetate was sequentially extracted with acetone and methanol. Each fraction was characterized for their antimicrobial activities (such as MIC) and their chemical constituents. The phenolic compounds, mainly gallic acid, present in each fraction was evaluated using HPLC technique and the Folin-Ciocalteu reagent. Gel containing ESDE was developed in the presence of sodium metabisulfite, sodium benzoate, and citrate buffer pH 3.0. The gel containing ESDE was further evaluated for its physical and chemical stability at 30 ± 2 °C, 75 ± 5 %RH for 1 month. Finally, the anti-acne efficacy of the gel containing ESDE was evaluated on 25 patients.

ESDE and its fractions contained more phenolic content (15.77-29.92%, w/w) than the commercially available emblica extract and its fractions. MIC of ESDE was 10 mg/mL while other fractions did not show obvious antibacterial activities against *S. aureus* ATCC 25923 and 29 clinical isolates due to their limited water solubility. Further analysis of data from previous study of the peak area and time profile of ESDE in citrate buffer pH 3.0 stored at 30 $\pm$ 2 ^{o}C for 1 month showed the increment of gallic acid and peak no.1, the decrement of peak no.2, 5 and 6 and the consistent of peak no.3. Interestingly, peak no.6 decreased dramatically within a week and the observed inhibition zone against *S. aureus* was also decreased. Gel containing emblica extract (GCE) was translucent with yellow color. The color was change from yellow to brown within 1 week and eventually dark brown within 1 month. During 1 month storage at 30 $\pm$ 2 ^{o}C and 75 $\pm$ 5 % RH, the pH was observed to be 3.24 $\pm$ 0.06, the viscosity changed from 1238 $\pm$ 75.60 to 1040 $\pm$ 21.79 cP and gallic acid content increased from 0.06 to 0.09 % w/w. The peak area and time profile of GCE was similar to that of ESDE in citrate buffer. GCE was tested its anti-acne efficacy for 1 month and 1% clindamycin gel was used as a positive control. The individual acne reduction from week 0 to week 4 was significantly different, $p<0.05$. At the end of each week, mean acne reduction between two groups was not different, $p>0.05$.

Department: Pharmaceutics and Industrial Pharmacy...Student's Signature……

Field of Study: …...Pharmaceutical Technology…….. Advisor's Signature……..…...

Academic Year: ……..…2012……..Co-advisor's Signature……

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ATCC	The American Type Culture Collection
^{o}C	degree Celsius
CFU	colony-forming unit
cP	centipoise
DMSO	Dimethyl sulfoxide
HPLC	high performance liquid chromatography
kg	kilogram
M	Molar
mg/mL	milligram per milliliter
min	minute
mL	milliliter
mL/min	milliliter per minute
mm	millimeter
N	Normal
nm	nanometer
pH	the negative logarithm of the hydrogen ion concentration
q.s.	sufficient quantity
R^2	coefficient of determination
RH	relative humidity
rpm	revolutions per minute
RSD	relative standard deviation
SD	standard deviation

v/v	volume by volume
w/w	weight by weight
µg	microgram
µg/mL	microgram per milliliter
µL	microliter

CHAPTER I

INTRODUCTION

Acne vulgaris is a disease of the pilosebaceous unit. The pathogenesis of acne vulgaris is believed to involve three steps including the over production of sebum, the accumulation of keratin leading to the raising of *Propionibacterium acnes* population and the inflammation (เพ็ญวดี ทิมพัฒนพงศ์, 2539). The development stage of acne can be divided into non-inflammatory acne, comedone and inflammatory acne, papules, pustules and nodulocyst (ปรียา กุลละวณิชย์ และ ประวิตร พิศาลบุตร, 2548). The gram-positive bacteria and gram-negative bacteria are believed to involve in the inflammation since they are cultivated from the pustule's content of the patients (Hassanzadeh, Bahmani and Mehrabani, 2008, Qa' dan et al., 2005). However gram-negative bacteria may not directly associate with the acne (Boni and Nehrhoff, 2003).

The treatments of acne depend on the development degree of acne lesion. The patient may be prescribed topical retinoid, azelaic acid or benzoyl peroxide for comedonal acne. For the mild to moderate papules and pustules the patient may be given adapalene in combination of benzoyl peroxide or benzoyl peroxide in combination of clindamycin. The patient may also be given azelaic acid, benzoyl peroxide, topical retinoid or systemic antibiotics. In severe papulopustular and moderate to severe nodular acne, isotretinoin is highly recommended. The female patients may be given the hormonal antiandrogens in combination with topical treatment or systemic antibiotics (Nast et al., 2012).

However, the topical anti-acne drugs could promote cutaneous side effect such as redness, scaling or burning. The use of antibiotics both topically and orally can promote the bacterial resistance (Hassanzadeh, P., Bahmani, M. and Mehrabani, D., 2008). Therefore, new substances including compounds from herbal origins are searched for their antibacterial activities in order to be used by patients with fewer side effects.

Phyllanthus emblica Linn. is a native plant widely distributing throughout Asia. The common name of this plant is amla, emblic, emblica, indian gooseberry or ma-kham-pom in Thai (นันทวัน บุณยะประภัสร และ อรนุช โชคชัยเจริญพร, 2539). Parts of this plant are used in traditional medicine in many Asian countries (Unander, Webster and Blumberg, 1990). Many research groups have reported biological activities of emblica such as antioxidant activity (Liu et al., 2008, Liu et al., 2008, Luo et al., 2009, Luo et al., 2011, Mayachiew and Devahastin, 2008, Scartezzini et al., 2006), enhancement of gastric mucus and hexosamine secretion (Bandyopadhyay, Pakrashi and Pakrashi, 2000), prevention of hepatic injury (Pramyothin et al., 2006), induction of fibroblast proliferation and production of procollagen (Fujii et al., 2008), antiviral and antimicrobial activities (Wang et al., 2009, Saeed and Tariq, 2007, Goud et al., 2008, Mandal et al., 2010, Ghosh et al., 2008). The development of emblica extract for using in a medicine, food and cosmetic industry had been studied exclusively in a few years ago (อุบลทิพย์ นิมมานนิตย์ และคณะ, 2550).

However, the instability of emblica fruit extract has been reported. The emblica extract solution in a solvent with pH 5.5 has been observed less stable than at pH 3.0 despite in a presence of antioxidant (Chinsuwan and Siritientong, 2008). Spray-dried emblica fruit extract is very hygroscopic. The adsorbed moisture

accelerated degradation of the spray-dried powder yielding brownish paste (อุบลทิพย์ นิมมานนิตย์ และคณะ, 2550). To get rid of the instability of the emblica extract, the proper solvent or packaging should be considered.

The cosmetic products containing emblica extract have been launched to the market. Most of them claim whitening properties. The herbal acne gel, Abhaibhubejhr, contains four plant extracts, including emblica extract but the function of emblica extract for this product does not claim clearly.

There are several cosmetics preparations that have been used for skin application such as emulsion, solution and gel. Aqueous topical gel is popular since it has a pleasant appearance, good feeling during and after application. Moreover it is suitable for loading of the water-soluble active ingredients. Gelling agent, which are usually used nowadays, are synthetic polymer such as HPMC, carbopol and poloxamer.

Poloxamer is a non-ionic synthetic block copolymer of ethylene oxide and propylene oxide. It is freely soluble in water and in alcohol. It is available in different grade varying from liquid to solid. All of the poloxamers have a thermoreversible property. Poloxamers are regarded as nontoxic and nonirritant materials and have been used as a dispersing agent, emulsifying and coemulsifying agent, solubilizing agent, tablet lubricant and wetting agent in many pharmaceutical formulations such as oral, parenteral and topical preparations (Rowe, Sheskey and Weller, 2003).

In this research, the antimicrobial activity of the emblica extract and its fractions was tested against *Staphylococcus aureus, Escherichia coli* and *Pseudomonas aeruginosa*. At the same time the emblica extract and its fractions were screened for their chemical constituents. The selected fraction was tested for its

stability in various medium including citrate buffer, phosphate buffer and water. Then gel containing emblica extract was prepared and tested for the physical and chemical stabilities at 30 $\pm$ 2 ^{o}C and 75 $\pm$ 5% RH for 1 month. Finally anti-acne efficacy of the emblica gel was compared with that of clindamycin gel on patient diagnoses with papules or pustules. The number of acne and the adverse effects were determined every 1 week during 1 month of study. The results were analyzed using the statistical technique.

Objectives

The overall objective for this study was to increase the economic value of the emblica fruit extract. The specific objectives of this study were as follows:

1. To prepare the emblica extract and its fractions
2. To characterize the chemical content and the *in vitro* antimicrobial activity of emblica extract and its fractions
3. To formulate gel containing emblica extract and characterize its chemical property and physical property
4. To study the stability of gel containing emblica extract
5. To evaluate the clinical efficacy of gel containing emblica extract as anti-acne

CHAPTER II

LITERATURE REVIEWS

I. *Phyllanthus emblica* L.

Phyllanthus emblica Linn. (Euphorbiaceae) or *Emblica officinalis* Gaertn. is a native plant widely distributing throughout Asia. The common name of this plant is emblica, amla, emblic, indian gooseberry or ma-kham-pom in Thai (นันทวัน บุณยะประภัศร และ อรนุช โชคชัยเจริญพร, 2539). Parts of this plant are used in folk medicine in many Asian countries (Unander et al., 1990). In India, fresh fruit is used for cooling effect, diuretic and laxative. In Thailand, bark has been used in a treatment of mouth infection and gastrointestinal disorders. Fresh fruit has been eaten for general fatigue, weakness and poor appetite. Moreover it has been used as antitussive and expectorant. Appearance of *P. emblica* is presented in Figure 1.

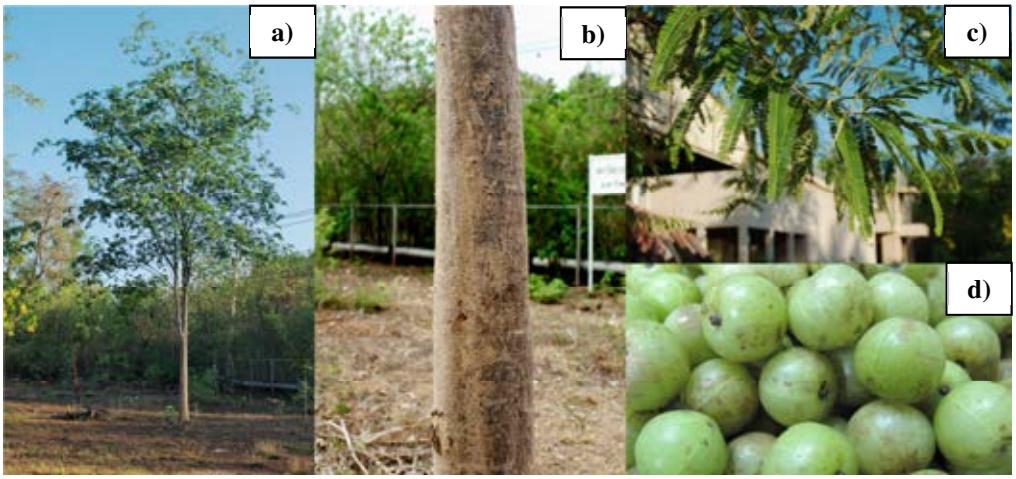


Figure 1 *Phyllanthus emblica* L.; a) Tree of *P. emblica,* b) Trunk, c) Leave, d) Fruits

Chemical constituents of emblica fruit extract

Ascorbic acid, polyphenol, hydrolysable tannins and flavonoids are found in emblica fruit extracts. Emblica fruit enriches with ascorbic acid (Scartezzini et al., 2006). The fruits which are processed according to Ayurvedic contain higher concentration of ascorbic acid than that in frozen or dried fruits (Jain and Khurdiya, 2004). Thus, a proper preparation method can prevent ascorbic acid from oxidation. The edible parts, pulp and peels, are a source for various phenolic acids, ascorbic acid and sugars. Examples of phenolic acids found in emblica pulp and peel include protocatechuic acid, p-hydroxy benzoic acid, vanillic acid, chorogenic acid, caffeic acid, syringic acid, p-coumaric acid, ferulic acid and sinapicnic acid (Kubola, Siriamornpun and Meeso, 2011). A number of phenolic constituents are also found in the aqueous acetone extract of fruit juice powder (Zhang et al., 2001). The gallate derivatives of mucic acid which are hydrolysable tannins, are not stable in aqueous solution and rapidly undergoes degradation giving rise to a series of degradation products. Hydrolysable tannins such as Furosin, corilagin, and geraniin are found in ethyl acetate fraction of emblica fruit extract (Kumaran and Karunakaran, 2006). Flavonoids i.e. quercetin, kaempferol and their glycosides are found in a methanolic fraction further extracted by ethyl acetate (Liu et al., 2008). Gallic acid is found at 0.59% from the freezed-dried extract of fresh juice (Sawant, Prabhakar and Pandita, 2010). The chemical structure of ascorbic acid and phenolic acid, phenolic compounds, hydrolysable tannins and flavonoids are presented in Figure 2-5.

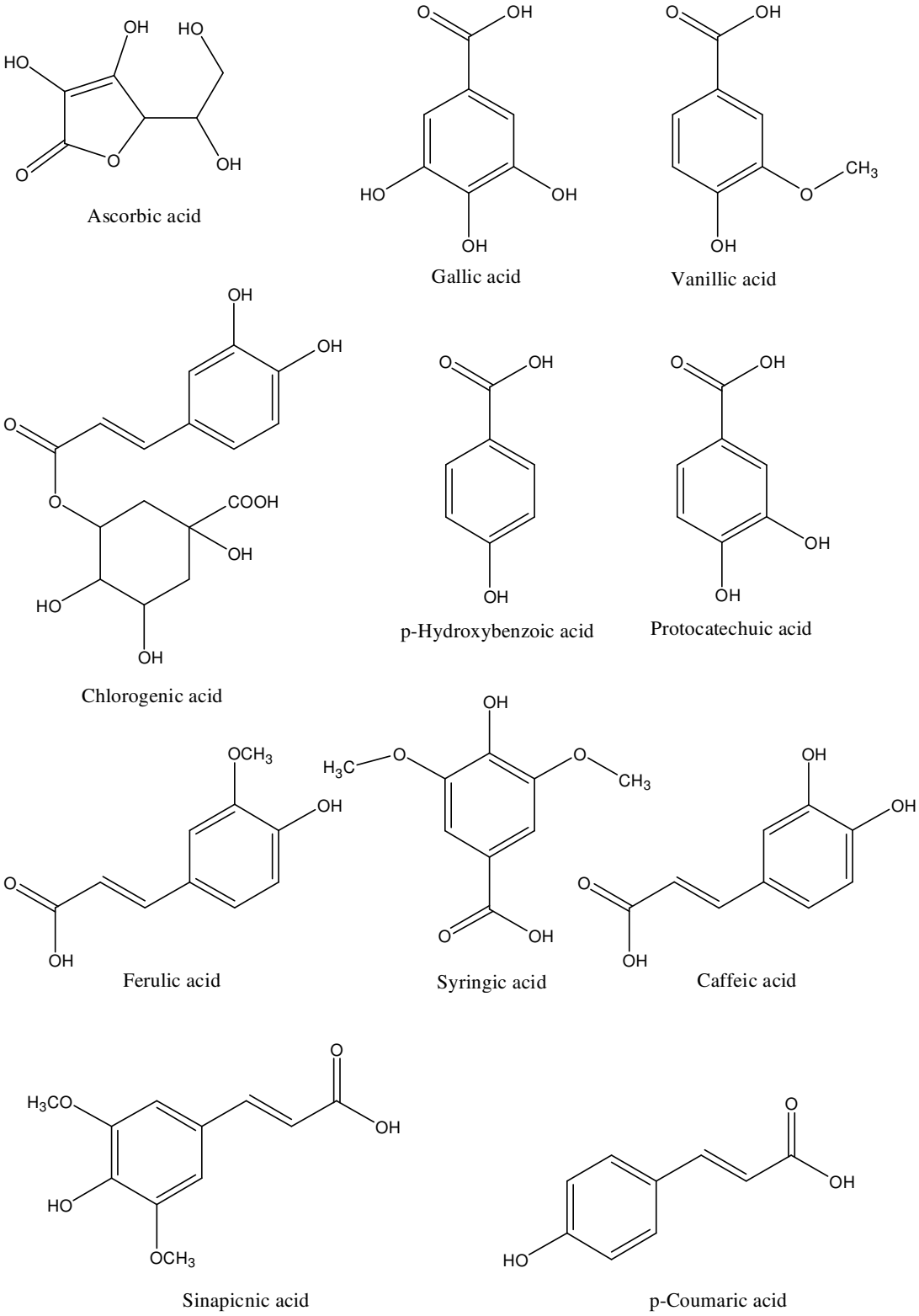
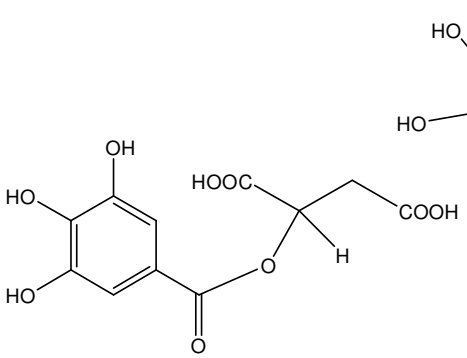
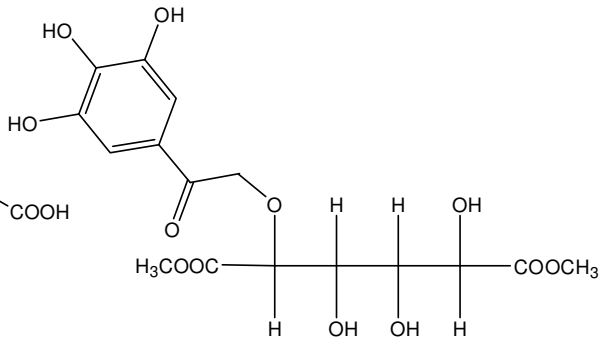


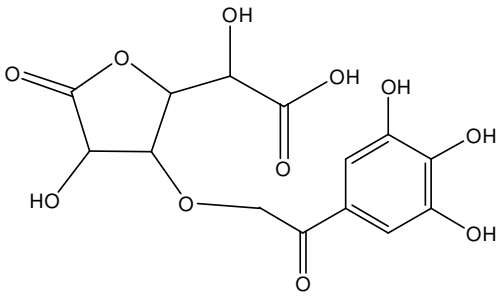
Figure 2 Ascorbic acid and phenolic acids in emblica fruit (redraw from Kubola et al. (2011) and Sawant et al. (2010))



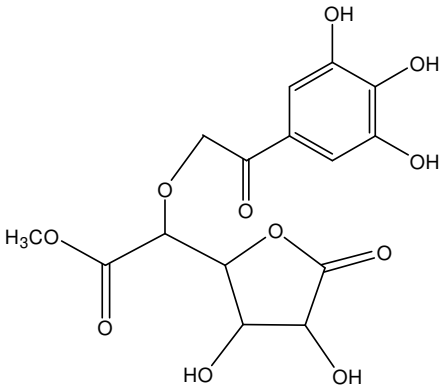
L-malic acid 2-*O*-gallate



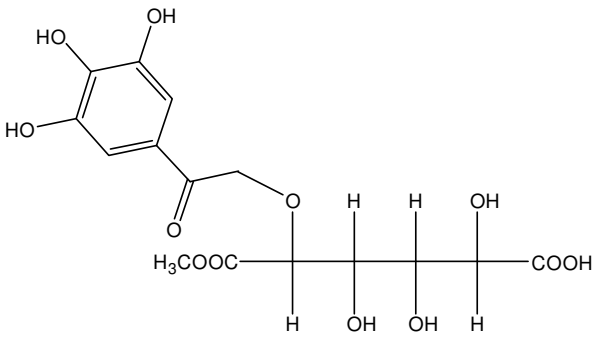
Mucic acid dimethyl ester 2-*O*-gallate



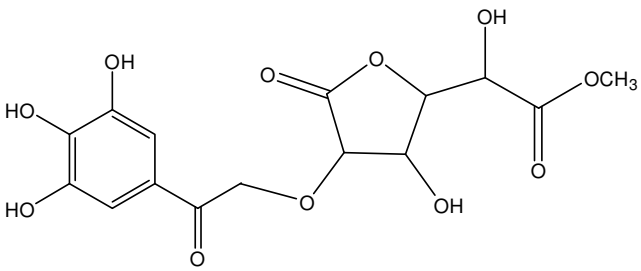
Mucic acid 1,4-lactone 3-*O*-gallate



Mucic acid 1,4-lactone 6-methyl ester 5-*O*-gallate

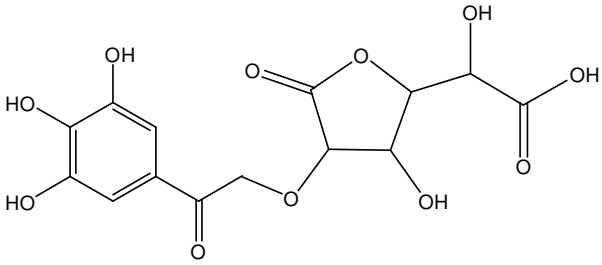


Mucic acid 1-methyl ester 2-*O*-gallate

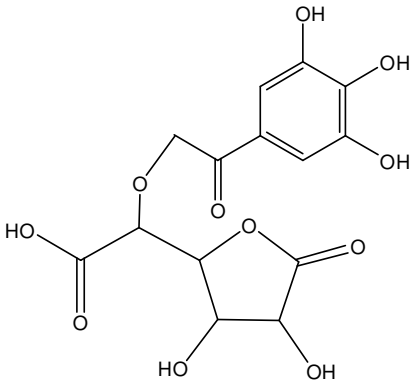


Mucic acid 1,4-lactone 6-methyl ester 2-*O*-gallate

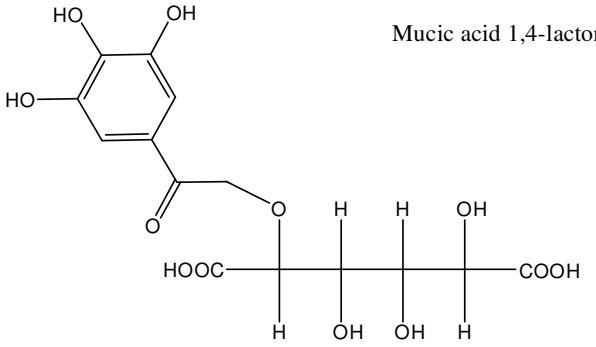
Figure 3 Phenolic constituents from emblica fruit juice (redraw from Zhang et al., 2001)



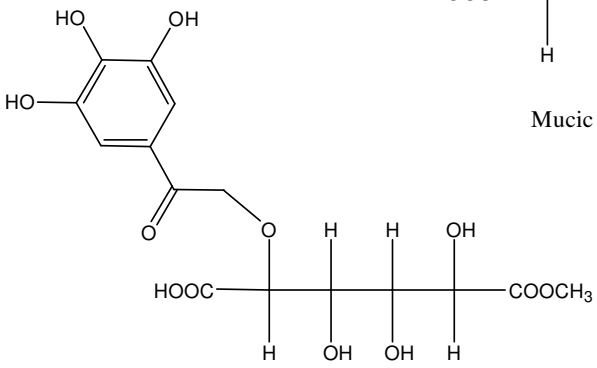
Mucic acid 1,4-lactone 2-*O*-gallate



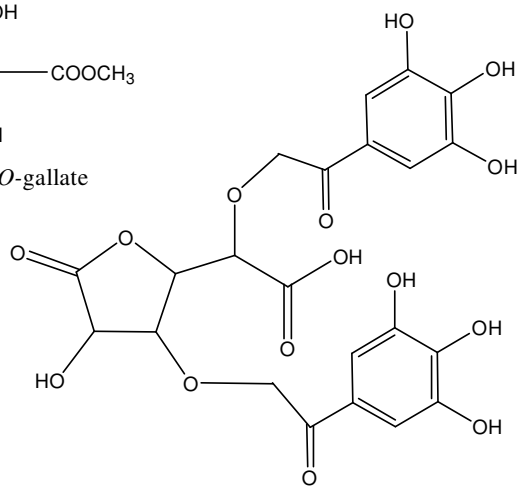
Mucic acid 1,4-lactone 5-*O*-gallate



Mucic acid 2-*O*-gallate



Mucic acid 6-methyl ester 2-*O*-gallate



Mucic acid 1,4-lactone 3,5-di-*O*-gallate

Figure 3 (cont.) Phenolic constituents from emblica fruit juice (redraw from Zhang et al., 2001)

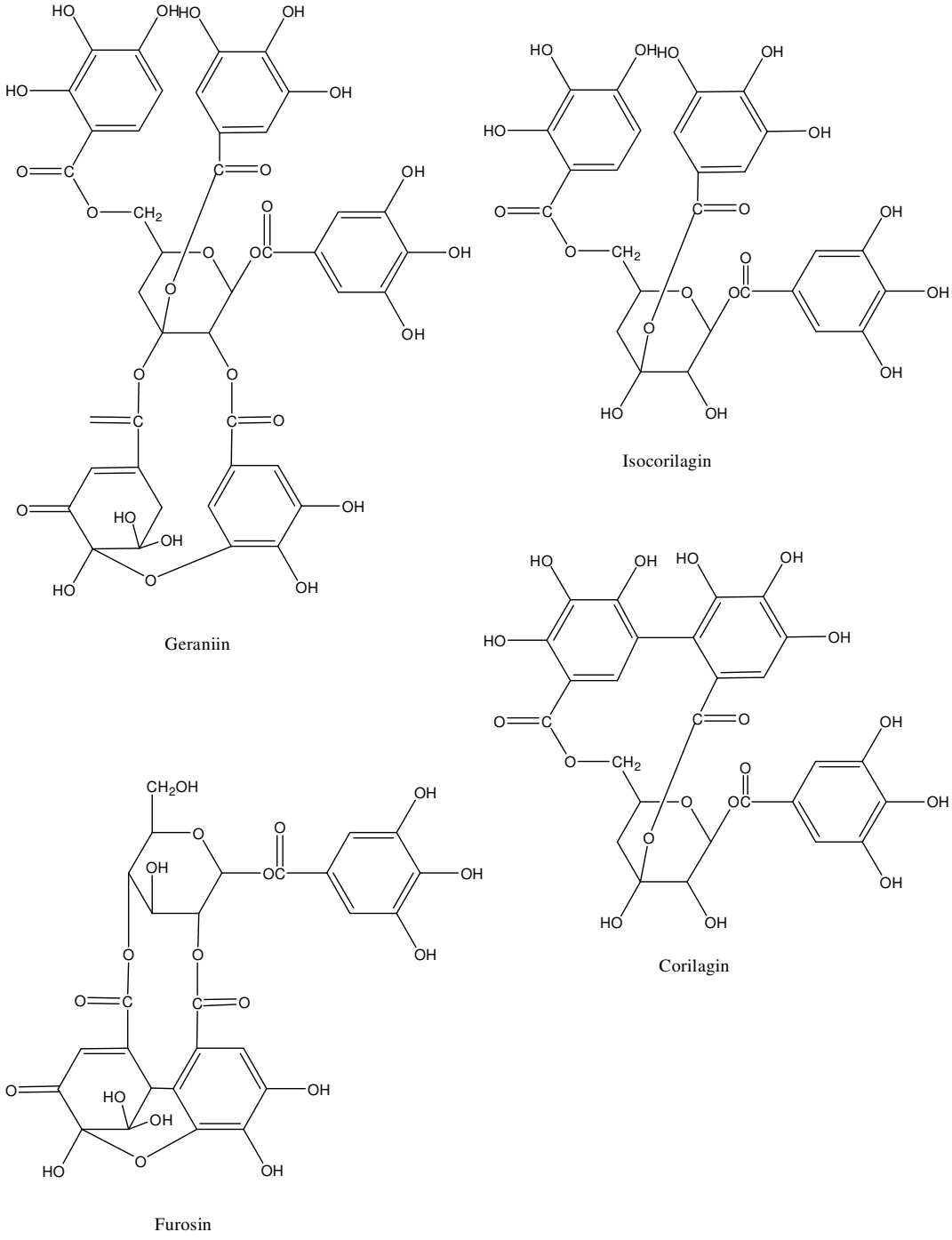
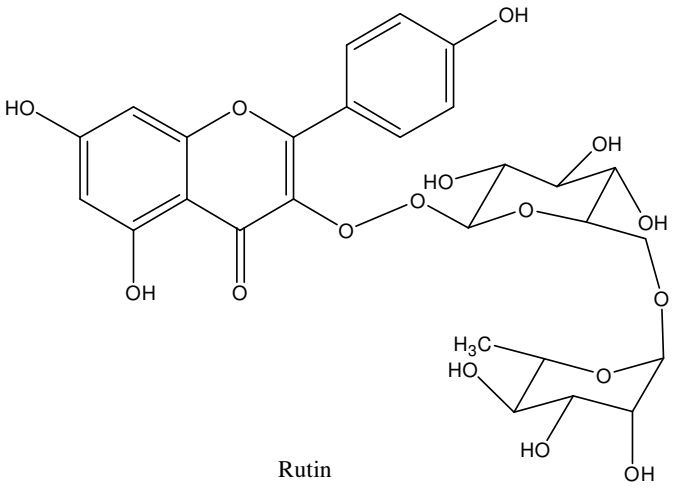
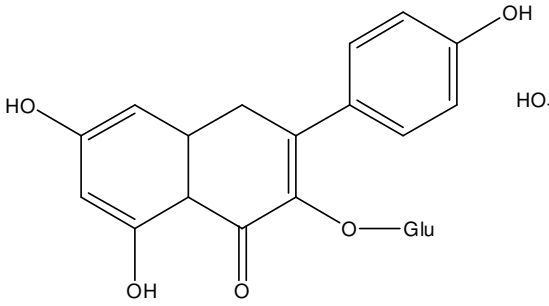


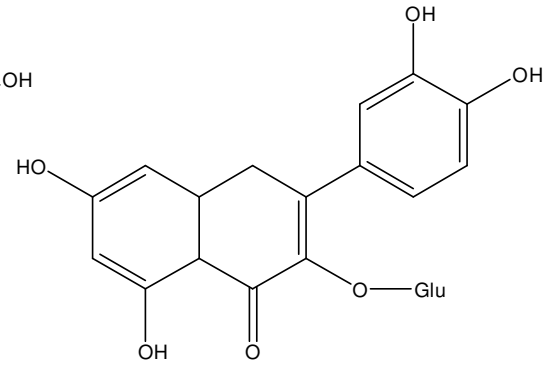
Figure 4 Hydrolysable tannins from emblica fruit extract (redraw from Kumaran and Karunakaran (2006) and Liu et al. (2008))



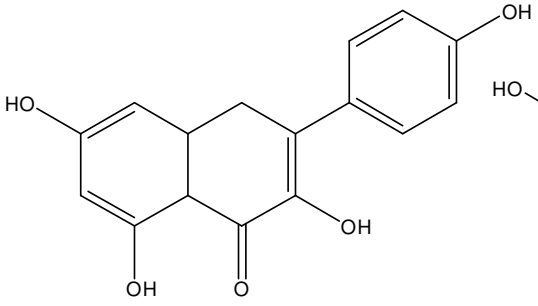
Rutin



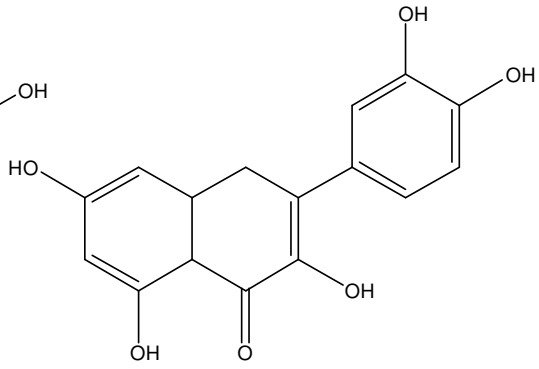
Kaempferol 3-β–D-glucopyranoside



Quercetin 3-β–D-glucopyranoside

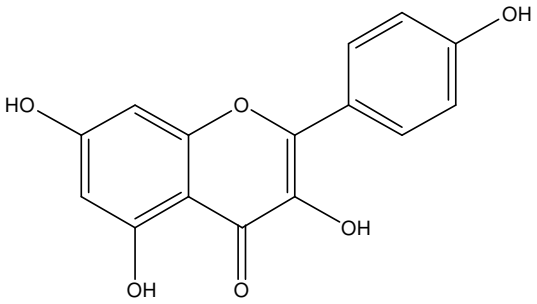


Kaempferol

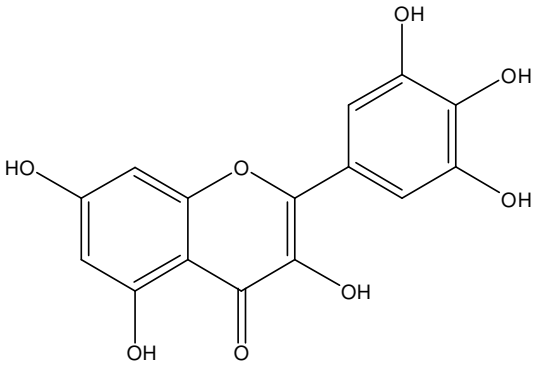


Quercetin

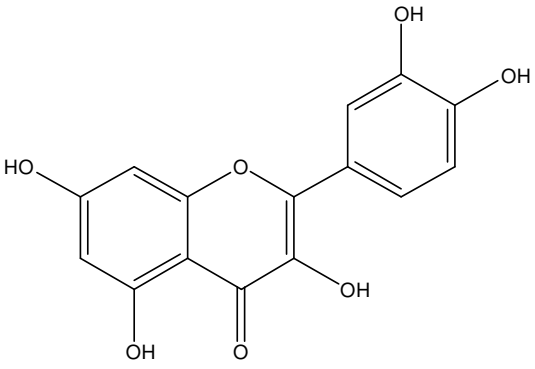
Figure 5 Flavonoids from emblica fruit extract (redraw from Liu et al. (2008) and Kubola et al. (2011))



Apigenin



Myricetin



Luteolin

Figure 5 (cont.) Flavonoids from emblica fruit extract (redraw from Liu et al. (2008) and Kubola et al. (2011))

The biological activity of emblica fruit extract

Many research groups have reported biological activities of emblica fruit extract such as antioxidant activity (Liu et al., 2008, Luo et al., 2009, Luo et al., 2011, Mayachiew and Devahastin, 2008, Scartezzini et al., 2006), enhancement of gastric mucus and hexosamine secretion (Bandyopadhyay et al., 2000), prevention of hepatic injury (Pramyothin et al., 2006), induction of fibroblast proliferation and production of procollagen (Fujii et al., 2008) and antiviral and antimicrobial activities (Wang et al., 2009, Saeed and Tariq, 2007, Goud et al., 2008, Mandal et al., 2010, Ghosh et al., 2008).

Phenolic compounds in methanolic or ethanolic emblica fruit extracts are reported to show antioxidant activity. However, comparison of the free radical scavenging activity of the extracts with antioxidant activity of quercetin or BHT is somewhat controversial (Liu et al., 2008). Oral administration of butanolic emblica extract shows the prevention of indomethacin induced gastric ulcer in rats because the extract enhanced gastric mucus formation and hexosamine secreation (Bandyopadhyay et al., 2000). Pretreatment of 50% ethanolic extract of fresh emblica fruit could prevent an increasing of hepatotoxic marker due to ethanol digestion in rats. In other words, emblica fruit extract could prevent the hepatic injury induced by ethanol (Pramyothin et al., 2006). The extract from Indian dried amla powder induces proliferation of fibroblast and production of procollagen (Fujii et al., 2008). Phyllaemblicin B from root of *P. emblica* inhibited Coxsackie virus B3 which is viral myocarditis by suppression of virus-induced apoptosis (Wang et al., 2009). The aqueous and methanolic leave extract of emblica shows antimicrobial activity against *Escherichia coli, Bacillus subtilis, Staphylococcus aureus, Proteus vulgaris* and

Enterobacter aerogenes (Ghosh et al., 2008). The aqueous infusion and aqueous decoction of emblica fruit disclose the antimicrobial activity against gram positive bacteria and *Candida albicans* (Saeed and Tariq, 2007). The methanolic extract of *P. emblica* whole plant material has antimicrobial activity against gram positive and gram negative bacteria (Goud et al., 2008). The ethanolic extract of emblica dried fruit shows antimicrobial against *S. aureus* with MIC of 13.97 mg/ml (Mayachiew and Devahastin, 2008). The combination of emblica extract and *Nymphae odorata* extract has a synergistic effect in antimicrobial activity against *S. aureus* (Mandal et al., 2010).

The problems of using emblica extract

The instability of emblica extract solution at pH 5.5 has been reported and showed that the extract solution is more stable at pH 3.0 in a presence of sodium metabisulfite (Chinsuwan and Siritientong, 2008). However, buffer effect on stability of aqueous emblica solution at pH 3.0 has never been reported. In addition, pale green-yellow solution of emblica extract quickly turns to brownish solution in the presence of light. Spray-dried emblica fruit extract is very hygroscopic. The adsorbed moisture accelerates degradation of the spray-dried powder yielding brownish paste (อุบลทิพย์ นิมมานนิตย์ และคณะ, 2550). Thus the spray-dried powder should be kept in a hermetically sealed container and protected from light. The factors in preparation of emblica powder affect the color and the ascorbic acid content (Methakhup, Chiewchan and Devahastin., 2005). High temperature drying process increases the difference of color from the beginning more than low temperature drying process. The causes of color change of the emblica powder are the degradation of ascorbic acid and

chlorophyll and the Maillard reaction. To prevent the cause of Maillard reaction, oxygen and light, the vacuum drying is introduced.

Phenolic compounds

Phenolics such as hydrolysable tannins, flavonoids and phenolic acid is a major chemical constituents presented in emblica fruit extract. It has been discovered relating to some biological activities such as antioxidant activity (Liu et al., 2008), nitric oxide radical scavenging (Kumaran and Karunakaran, 2006) and antimicrobial acitivity (Mayachiew and Devahastin, 2008). The types of extracted solvent, pH and temperature could bring the reaction during the extraction process of hydrolysable tannins (Mueller-Harvey, 2001). Unlike the condensed tannins, the depside bonds of hydrolysable tannins cleave with neutral pH methanol extraction. But when acidic methanol, pH < 3, is used the bonds are not cleave. The thermal process of tomatoes affects the stability of phenolic compounds both pros and cons (Jacob, Garcia-Alonso and Ros, 2010). The beneficial of using thermal process is increasing the number of antioxidants but it should avoid extension of thermal process to prevent the degradation of antioxidant compounds. High pH of the solvent induces irreversible degradation of caffeic acid, chlorogenic acid and gallic acid (Friedman and Jurgens, 2000). The chemical structure of the phenolic acid is destroyed resulting in the change of UV chromatogram. But high pH does not affect the permanent change of catechin, epicatechin and rutin. These compounds have two benzene rings and the electron delocalization in the molecule prevents the formation of quinone intermediates.

II. Acne

Acne vulgaris is a disease of sebaceous gland. The pathogenesis of acne vulgaris is believed to involve three steps. Firstly, an increase in sebum production results from enhancement of androgen production as one approach puberty. Secondly, an increase in keratin production gives rise to keratin accumulation and comedone formation in follicles, which is the first stage of acne vulgaris. Thirdly, anaerobic condition in the sebaceous gland promotes growth of *Propionibacterium acnes* (*P. acnes)*, a normal flora in the sebaceous gland. It is believed that *P. acne* causes inflammation by production of cytotoxins which are chemoattractants for polymorphonuclear leukocytes (PMN) and production of lipases which can digest triglycerides in sebum to free fatty acids causing skin irritation and inflammation (เพ็ญวดี ทิมพัฒนพงศ์, 2539). The inflammatory acne can be classified as papules, pustules and nodulocyst (ปรียา กุลละวณิชย์ และ ประวิตร พิศาลบุตร, 2548).

Both gram-positive and gram-negative bacteria are also reported to take part in acne ethiology. Gram-positive bacteria including *S. epidermidis, S. aureus* and *Micrococcus spp.* have been cultivated from the pustular and nodulocystic skin lesions (Hassanzadeh, Bahmani and Mehrabani, 2008, Qa' dan et al., 2005). *S. aureus* is the most frequent isolated bacteria found in acne patients. It may directly associate in acne development. Gram-negative bacteria such as *Klebsiella spp., E. coli, Enterobacter spp., Proteus spp.* and *Pseudomonas aeruginosa* have been cultivated from pustules content of the patients who has a Gram-negative folliculitis resulting from long-time medicated with oral antibiotics (Neubert, Jansen and Plewig, 1999). The outgrowth of the gram-negative normal flora is believed to be a result of

reduction or eradication of gram-positive normal flora. However it is not consider as a variant of acne (Boni and Nehrhoff, 2003).

Treatment of acne

In a treatment of acne vulgaris and acneiform eruptions such as folliculitis, a topical drug, an oral drug or a combination of topical and oral drugs is prescribed depending on the types and severity of acne (Wolff et al., 2008). The oral medicines are classified as antibiotics, drugs for suppressing sebum secretion, and non-steroidal anti-inflammatory drugs (NSAIDs). While the topical anti-acne drugs are classified as anti-comedone, anti-bacterial and anti-inflammatory drugs. The topical anti-acne drugs could promote skin irritation, redness or dryness. The long term use of antibiotics both topically and orally can promote the bacterial resistance. Clindamycin topical solution was reported to be absorbed leading to pseudomembranous colitis (Barza et al., 1982). Therefore, new substances including compounds from herbal origins are searched for their antibacterial activities in order to be used by patients with fewer side effects.

III. Gel

Gel is a semi-solid preparation. Aqueous topical gel gains its popularity due to its pleasant appearance, good feeling during and after application. Gel viscosity makes its applicable and slowly release active ingredient(s) to skin. Thin film of gel on skin also has an occlusive effect. As a result, gel prevents water loss and improves skin moisture content. Gelling agent, which are usually used nowadays, are synthetic polymer such as HPMC, carbopol and poloxamer.

Poloxamer

Poloxamer is a non-ionic synthetic block copolymer of ethylene oxide (EO) and propylene oxide (PO), $HO(C_2H_4O)_a(C_3H_6O)_b(C_2H_4O)_aH$ (Rowe et al., 2003). The structure formula of Poloxamer is shown in Figure 6. It is available in different form varying from liquid to solid depends on the number of monomer of ethylene oxide (a) or propylene oxide (b). It is freely soluble when use water and 95% ethanol as a solvent. The polyethylene oxide region is hydrophilic whereas the polypropylene oxide is hydrophobic. Poloxamers are regarded as nontoxic and nonirritant materials and have been used as a dispersing agent, emulsifying and coemulsifying agent, solubilizing agent, tablet lubricant and wetting agent in many pharmaceutical formulations such as oral, parenteral and topical preparations (Kojarunchitt et al., 2011, Ricci, et al., 2002).

When Poloxamer 407 is dissolved in aqueous solution then increases temperature, the PO block aggregates due to the dehydration and its hydrophobic property to form a core and EO blocks are hydrated and swelled then the micelle structures are formed. Continue increasing temperature, the micelle structures connect each other to form network and then gel structure is formed (Dumortier et al., 2006). Aqueous solution of Poloxamer 407 shows a thermoreversible property. The association of Poloxamer in water is shown in Figure 7.

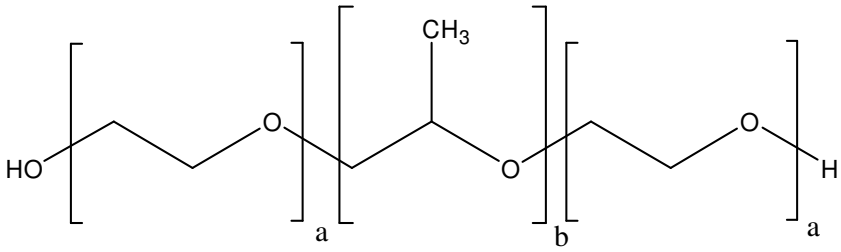


Figure 6 Structure formula of Poloxamer (redrawn from Rowe et al., 2003)

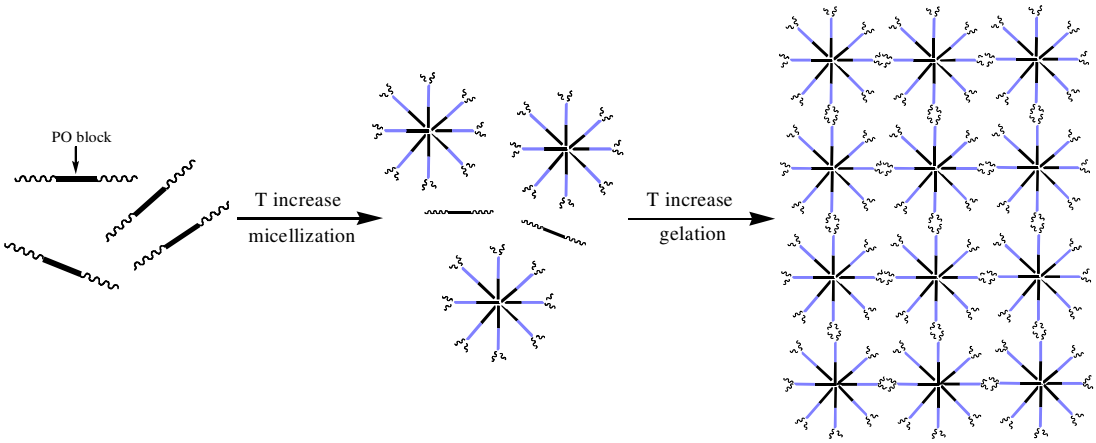


Figure 7 Association of poloxamer 407 in water (redrawn from Dumortier et al., 2006)

CHAPTER III

MATERIALS AND METHODS

Materials

Two different Emblica fruit extract powders were used in the screening study. Emblica extract Lot No.TS8DPHE71A was purchased from Specialty Natural Products Co., Ltd., SNP, Thailand and In-house emblica fruit extract was prepared according to a method previously published by Nimmannit et al, 2007.

The gram positive bacteria, *S. aureus* ATCC 25923 and 29 clinical isolates and the gram negative bacterias, *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853, were kindly provided by Assoc. Prof. Dr.Pintip Pongpech, Department of Biochemistry and Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Throughout the study bacteria were grown and tested on Triptic Soy Bean agar, Lot 1074969, and Muller Hinton agar, Lot 1073002, BD Difco^{TM}, USA.

The C18 column and C18 guard cartridge (HyperClone 5u ODS C18 250x4.6 mm) were obtained from Phenomenex, USA. The Nylon Syringe filter (diameter 13 mm) was supplied from Scientific Promotion, Thailand. Filter paper Whatman® No.1 and No.5 was supplied from Whatman®, UK.

Methanol (AR grade, HPLC grade), Acetone (AR grade), Dimethyls Sulfoxide (DMSO, AR grade) and Ethyl acetate (AR grade) were obtained from Labscan Asia,

Thailand. Folin Ciocateau's reagent, Gallic acid and Trifluoroacetic acid were obtained from Sigma-Aldrich, USA. Sodium dihydrogen phosphate ($NaH_2PO_4.H_2O$) and Sodium citrate $C_6H_5Na_3O_7.2H_2O$ was obtained from Merck, USA. Phosphoric acid (H_3PO_4) was obtained from J.T.Baker, USA. Citric acid ($C_6H_8O_7.H_2O$) was obtained from Univar, USA. Poloxamer 407 was obtained from BASF, Germany. Propylene glycol, Sodium benzoate and Sodium metabisulfite were used as received.

Instruments

- High-Performance Liquid Chromatography System (LC10, Shimadzu, Japan) equipped with
    - A LC-10AD vp Liquid Chromatograph (Pump)
    - a SCL-10A vp System Controller
    - a SPD-10A UV-Vis Detector
    - a SIL-10AD vp Autosampler
    - a CTO-10A Column oven

    and Shimadzu Class VP Software
- pH meter model 420A (ORION, USA)
- pH meter model PB 20 (Sartorius, Germany)
- Balance , Mettler AG 285 (Mettler Toledo, Switzerland)
- Balance , Mettler PG 403-S (Mettler Toledo, Switzerland)
- UV-Vis spectrophotometer Model 160 A (Shimadzu, Japan)
- Rotary evaporator (Rotavapor R-200, Buchi, Switzerland)
- Pipettboy acu (IBS Integra Biosciences, Switzerland)
- Mechanical Pipettor 1-5 mL (Biohit, Finland)

- Mechanical Pipettor 200-1000 µl (Biohit, Finland)
- Mechanical Pipettor 20-200 µl (Biohit, Finland)
- Vortex mixer (Labnet, USA)
- Vitex Colorimeter (BioMerieux, France)
- Autoclave model HA-3D (Hirayama, Japan)
- Incubator (Mammert, Germany)
- Hot Air Oven (Heraeus, Germany)
- Ultrasonic cleaner, CAVITATOR® (Mettler Electronics corp., USA)
- Gas Burner
- Soxhlet apparatus
- Electronic Digital Caliper

Methods

1. Preparation of emblica extract and its fractions

Emblica fruit extract powder (EFEP) from 2 different sources; i.e. SNP, Thailand and in-house emblica extract (ESDE) were employed. Each of them was subjected to the following extraction procedure resulting in 5 different fractions.

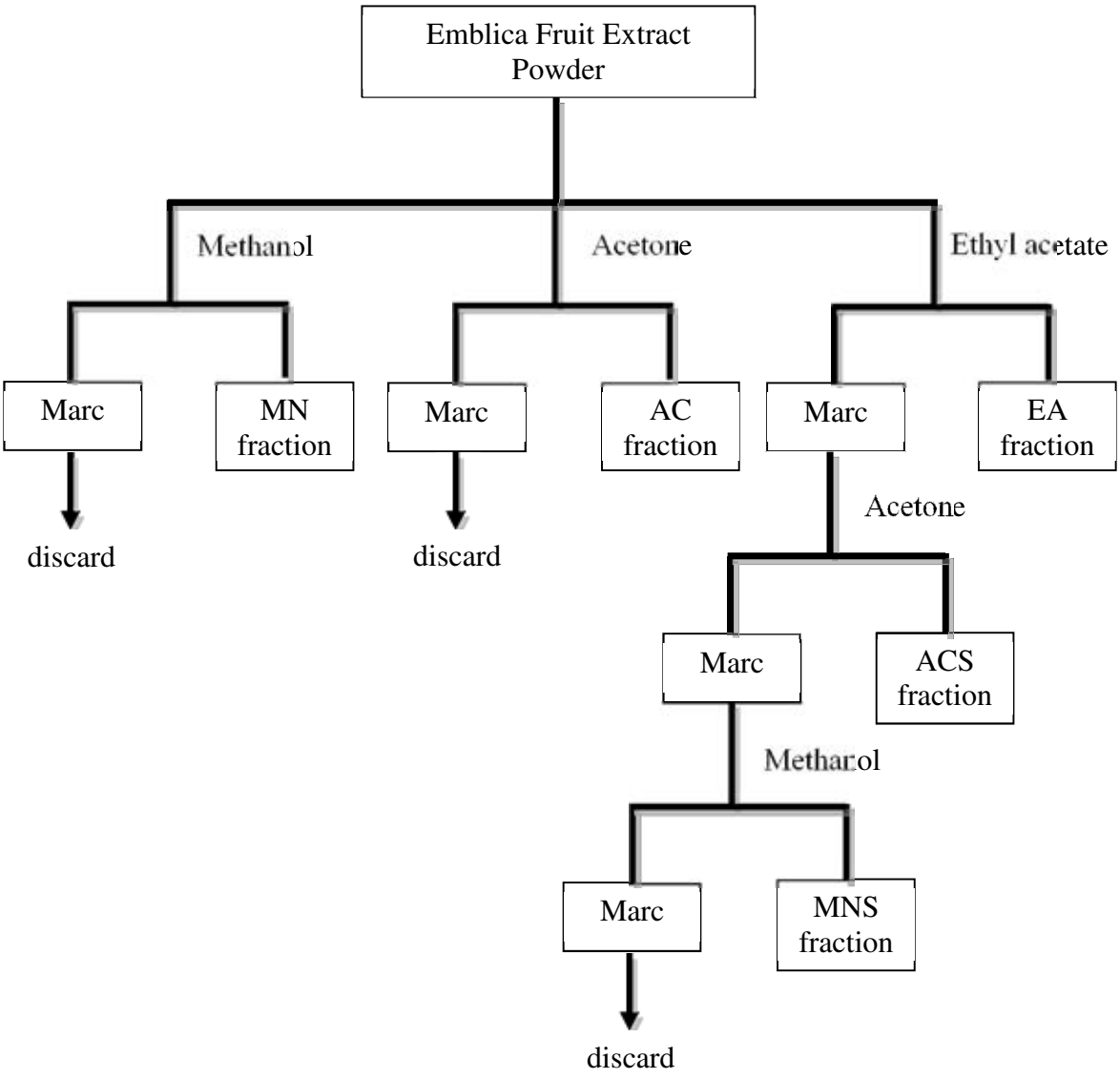
The in-house emblica extract was prepared according to a method published by Nimmannit et al. (2007). Briefly, emblica fruit were purchased from Ta-laad-thai market in November, 2010. The fruits were rinse with water, crushed and filtered through a cotton sheet. Water was then added to the marc and filtered through a cotton sheet. Generally, 1 L of water was added to 1.5 kg of initial fruit weight and 0.5 L of water was added to 1 kg of marc from the first extraction. The filtrate from

these 2 steps was pooled prior to spray dry. During the spray dry process, inlet and outlet temperature were set at 175 and 95 ^{o}C with feeding rate of 24 L/hour. Percent yield of the emblica spray-dried powder (ESDE) was calculated based on weight of the obtained ESDE and the weight of emblica fresh fruit.

The emblica fruit extract powder was separately extracted with acetone, ethyl acetate or methanol by soxhlet apparatus (Scheme 1). The marc from ethyl acetate extraction was subsequently extracted by acetone and methanol. The fractions were dried under vacuum using a rotary evaporator giving rise to acetone (AC), ethylacetate (EA), methanol (MN), subsequent acetone (ACS) and subsequent methanol (MNS) fractions. Percent yield of each fraction was calculated according to Eq.1 and reported.

$$\% \text{ yield} = \frac{\text{weight of the fraction}}{\text{weight of EFEP}} \times 100 \qquad \textbf{Equation 1}$$

Where weight of the fraction is the fraction weight obtained after the drying process, weight of EFEP is the initial weight of EFEP



Scheme 1 Extraction procedure of AC, EA, MN, ACS, MNS fractions

2. Characterization of emblica extract and fractions

2.1 HPLC Characterization

The Shimadzu HPLC was used to characterize SNP, ESDE and their fractions. The HPLC condition was modified from the previously reported method (Nimmannit, et al., 2007). The modified HPLC method could accurately quantify gallic acid, a marker, present in the extracts and fractions (Appendix B).

The separation was done on two Phenomenex C18 reversed phase column i.e. Luna 5u C18(2) 100A, 4.6x150 mm or HyperClone 5u ODS C18 120A, 4.6x250 mm. Both are different in the pore size of silica and the column length. Trifluoroacetic acid (TFA) was diluted to 0.05, 0.1, and 0.5% with water. The series of the ratio between trifluoroacetic acid and methanol were used as a mobile phase. A flow of the mobile phase through the column was set at 0.5 or 1.0 mL/min. In this study, the best conditions were reported.

Column	: C18 (Phenomenex HyperClone 5u C18 120A, 4.6x250 mm)
Mobile phase	: 0.05% TFA:Methanol (95:5)
Injection volume	: 20 µl
Flow rate	: 1 ml/min
Column oven	: 30 ^{o}C
Detection	: 270 nm
Run time	: 15 minute

The ESDE or its fractions (MN, AC, ET, ACC and MNC) were separately dissolved in methanol. The final concentration of ESDE, MN and MNS each samples were around 2000 µg/mL whereas ET, AC and ACC were prepared at around 200 µg/mL. Then each sample was filter through a nylon membrane with pore size 0.45 µm diameter of 13 mm prior to analyze by HPLC. The column was maintained at 30 ^{o}C with an analytical wavelength of 270 nm. Chemical composition of SNP, ESDE and their fractions were characterized by comparison of peak pattern and relative

retention time of the peaks with those of standard gallic acid. Standard gallic acid solutions were prepared by dissolving gallic acid to final concentrations of 4, 8, 16, 24, 32 µg/mL. Gallic acid content in term of gallic acid equivalent (GAE) was calculated from the regression equation constructed from the standard curve of gallic acid.

The chemical composition in ESDE was further analyzed by LC-MS. The test was done by the Central Instrument Facility, Faculty of Science, Mahidol University, Bangkok, Thailand. The HPLC condition was similar to the condition previously used to detect the chemical composition in SNP and its fractions.

2.2 Determination of total phenolic compounds

Total phenolics in the emblica crude extract and fractions were determined using Folin-Ciocalteau reagent (Singleton and Rossi, 1965), a complex of phosphotungstate and phosphomolybdate. Under basidic conditions, the phenolic proton is turned into the phenolate anion which has a reducing power against Folin-Ciocalteu reagent giving rise a blue color product (Huang, Ou and Prior, 2005). One milliter of sample solutions was reacted with 5 mL of a mixture of Folin-Ciocalteau reagent: water (1:10 v/v). After allowing the solution to stand for 3 to 8 minutes, 4 mL of 7.5% sodium carbonate was added and well mixed. The reaction mixture was kept at room temperature in the dark for 2 hours. Then the absorbance of the reaction mixture was determined using a UV-spectrophotometer with an analytical wavelength of 747 nm. Gallic acid was used as a positive control. Gallic acid standard solutions were dissolved in water and diluted to have concentrations of 10, 20, 40, 60, 80 and 100 µg/mL. ESDE and their corresponding fractions were separately dissolved and

diluted in water to make samples with a final concentration of around 100 µg/mL. Water was used as a blank solution. The total phenolics in ESDE and fractions were calculated from the regression equation of the standard curve as gallic acid equivalent (GAE, µg/mL) and reported as %phenolic compound present in the ESDE and its fractions. The absorbance values obtained from standard gallic acid solutions were plotted against its corresponding concentrations in order to construct a standard curve.

2.3 Screening test for antimicrobial activity

2.3.1 *In vitro* antimicrobial activity of the emblica crude extract and fractions

In vitro antimicrobial activity of ESDE and fractions will be determined using a modified cup plate method (Ghosh et al., 2008). Briefly, the well isolated colonies of the bacterial strains were inoculated into 0.85% normal saline solution to obtain the turbidity equal to McFarland standard solution no. 0.5 which is about 1 x 10^8 CFU/mL. Each bacterial suspension was spread throughout the entire surface of Muller Hinton agar (MHA) using sterile cotton swab. The solution of spray dried extract in water at a concentration of 25 mg/mL and of various fractions in 100% DMSO at a concentration of 50 mg/mL was prepared. Then 300 µl of the solution were added into the sterile stainless steel cylinder cups (diameter 6 mm), previously placed on the surface of the inoculated MHA plates. The 100% DMSO was used as the control. After the plates were incubated at 35 ^{o}C for 18 hours, the inhibition zone sizes surrounding the cylinder cup were measured.

2.3.2 Antimicrobial activity of ESDE against *S. aureus*

To determine the inhibition of ESDE against *S. aureus*, a modified disc diffusion method was used (Ghosh et al., 2008).

Twenty microliters of sample solution or standard solution was impregnated to a sterile 6 mm paper disc. The impregnated paper discs were left at room temperature to dry out the solvent prior to use. Tetracycline (TC) and clindamycin (CM) were used as a positive control.

The well isolated colonies of bacteria were suspended in 0.85% normal saline solution and adjusted to equal McFarland standard solution no. 0.5 which is about $1x10^8$ CFU/mL. The suspension was spread over the MHA plate thoroughly with a sterile cotton swab. Then discs containing sample or standard were placed on the top surface of MHA plate. The clear zone sizes (mm) were measured after incubated at 35^oC for 18 hours.

2.3.3 MIC determination of ESDE for *S. aureus*

Certain amount of ESDE, 0.1, 0.5, 1, 2.5, 5 and 10 mg/mL in media, was added into the molten Muller Hinton agar at temperature about 50 ^oC and mixed gently. Twenty-five milliliters of the premix MHA was poured to a sterile petri dish and solidified at room temperature.

The well isolated colonies of bacteria were suspended in a 0.85% normal saline solution and adjust to equal McFarland standard solution no.0.5 which is about $1x10^8$ CFU/mL. Three hundred microliters of the bacterial suspension was pipetted into a sterile well plate. A sterile replicator was dipped in the well plate to reach the bacterial suspension then place gently on the top surface of MHA plate which had

been inoculated with bacterial suspension. Two MHA plates without sample were used as a pre- and post-control. The plates were incubated at 35°C for 18 hours. The MIC value was determined from the lowest concentration that had no bacterial growth visibly.

3. Development of gel containing emblica extract

3.1 Stability study of ESDE in solutions

Three and a half grams of ESDE was dissolved in water adjusted pH to 3 with 1 N HCl, 0.15 M citrate buffer (pH 3.0), or 0.15 M phosphate buffer (pH 3.0) in the presence of 1% sodium metabisulfite and 0.1% sodium benzoate. The samples were adjusted to the volume (50 mL) with the same solvent. The samples were kept in glass vials, purged with nitrogen then tightly sealed with rubber stoppers and aluminium seals, and stored at 30 °C in the dark for 1 month. pH values of each samples were measured at the beginning and at the end of the study with a pH meter. Every week the samples were evaluated for appearance, color change. At day 0, 1, 2, 4, 6, 8, 18, 22 and 29, the samples were withdrawn and evaluated the chemical constituents using the HPLC method mentioned in 2.1. ESDE in water at the same concentration in the presence of 1% sodium metabisulfite and 0.1% sodium benzoate was used as a control.

Solutions of ESDE in water were prepared at 4 and 7 % w/w in the presence of 1% sodium metabisulfite. The solutions were stored at temperature 30 °C in the dark for 4 and 7 days. Then the samples were withdrawn and tested their antimicrobial activity against 5 strains of *S. aureus*; ATCC 25923, clinical isolates number 592, 577, 580 and 571 using the zone inhibition technique mentioned in 2.3.1.

In this study, the clinical isolate number 592 and 577 were randomly selected as representatives of *S. aureus* susceptible to TC while the clinical isolate number 580 and 571 were randomly selected as representatives of *S. aureus* resistant to TC.

3.2 Formulation of gel containing ESDE

3.2.1 Determination of suitable ESDE concentration in the preparation

ESDE solutions in water were prepared at 4, 7, 10, 15 and 20% w/w. The samples were tested for antibacterial activity against 4 strains of *S. aureus* using the disc diffusion technique. In this study, 2 sensitive and 2 resistant strains to TC were employed. *S. aureus* ATCC 25923 and the clinical isolate number 592, which was randomly selected, were representatives of *S. aureus* susceptible to TC. The clinical isolates number 571 and 580 were also randomly selected and were representatives of *S. aureus* resistant to TC. The samples were evaluated for their antimicrobial activity according to the zone inhibition test mentioned in 2.3.1.

3.2.2 Preparation of gel containing ESDE (GCE)

Gel containing emblica extract was prepared according to the preparation technique reported by Chinsuwan and Siritientong (2008) with slight modifications. The formula is shown herein. Poloxamer 407 was dispersed with propylene glycol in a glass mortar. A mixture containing 0.15% sodium metabisulfite in 0.15 M citrate buffer pH 3 was used as a solvent. ESDE was dissolved in the solvent and sonicated for 10 minutes. The mixture was then centrifuged a 5000 rpm for 10 minutes. The supernatant was decanted and further filtrated through a Whatman no.5 filter paper. Sodium benzoate was separately dissolved in the solvent. Portions of the filtrate and

sodium benzoate solution were added into the Poloxamer 407 and mixed well in the glass mortar. The mixture was transferred into a beaker. The motar was rinsed and the mixture was adjusted to the final weight with the solvent.

The mixture was kept at 4 ^{o}C in the dark overnight in order to minimize air bubble in the preparation. The gel was formed after the temperature of the mixture reached room temperature. Blank gel was used as a control. The gel preparation was done in triplicate.

	Percentage (% w/w)
Poloxamer 407	18
Propylene glycol	10
Emblica extract	10
Sodium benzoate	0.15
0.15% Sodium metabisulfite in citrate buffer pH 3.0	q.s. to 100

The obtained gels were stored at 30 $\pm$ 2 ^{o}C, 75 $\pm$ 5 % RH in the dark for 1 month and test for its physical and chemical stability and antimicrobial activity. At day 0, 7, 14, 21 and 21, the samples were taken and determined the appearance. At day 0, 5, 7, 14, 20 and 27 the samples were evaluated the pH. At day 0, 7, 13, 21 and 27 the samples were determined viscosity. The chemical stability was analyzed at day 0, 5, 7, 14, 21 and 28 by using HPLC technique as mentioned in 2.1. The antimicrobial activity of GCE was evaluated and compared with an antibacterial activity of commercial anti-acne gel, 1% clindamycin gel, using the zone inhibition test as mention in 2.3.1. At day 0 and day 28, thirty strains of *S. aureus* were included

in the test but the other tested dates only 5 strains, *S. aureus* ATCC 25923, two sensitive to TC strains and two resistant to TC strains were employed.

4. Efficacy testing of emblica gel

A 4-week double-blind, randomized, parallel design was designed in order to compare anti-acne efficacy of the GCE with 1% clindamycin gel, a commercially available preparation, on 25 volunteers at Siriraj Hospital, Bangkok, Thailand. Clinical improvement over 4 week period was reported as percentage of decrease in acne number and patient's skin conditions using Global Acne Assessment Score (GAAS).

The study protocol was approved by institutional review board (IRB) of Mahidol University at the Faculty of Medicine Siriraj Hospital, Mahidol University, Bangkok, Thailand. Thai male or female patients aged over 18 years with mild to moderate acne, defined as with more than 10 papules or pustules lesions was recruited. The volunteers were clearly informed about the study protocol, asked to read the written protocol and asked to sign consent form. The exclusion criteria were use of cosmetic products such as alcoholic toner and abrasive cleanser, use of anti-acne products within 2 weeks prior to and during the study, a history of allergic reaction, a history of hypersensitivity to clindamycin, administration of isotretinoin in the past 6 months, administration of hormone in the past 12 weeks, dermatologic conditions interfering with the treatment, a career that exposes to sunlight for longtime, pregnant or nursing, wish to terminate from study or violation of the study protocol.

Severity of acne in each patient was assessed by a physician according to Global Acne Assessment Score (GAAS). Then, the patients were randomized divided into two groups so that each group contains patients with the similar severity. One group was given the clindamycin gel and the other group was given GCE. GCE or 1% clindamycin gel were packaged in 5 mL HDPE tube with identical blinded labeling. Since the GCE was yellow gel while the clindamycin was a clear gel. Researcher was responsible for handling out the test products. Patients were asked to use cotton swab or cleaned finger to apply given gel on acne lesions twice daily after cleaning their faces with Cetaphil® gentle skin cleanser. The patients were received freshly prepared product every week. The physician assessed the acne lesions and count number of acne at the end of the first, second, third and fourth weeks. Skin adverse effect was also recorded. Anti-acne efficacy of each preparation was investigated by paired t-test. Anti-acne of both gels was compared by t-test.

Data evaluation

The data from the experiments were calculated for mean and standard deviation. The comparison of number of acne and the patient's skin condition (GAAS) from the beginning and at the end of the study was analyzed using Student paired t-test. Percent decreased in acne number between two groups were compared by t-test. The confidence interval for statistical analysis of this experiment is 95% which p-value less than 0.05 is considered as statistical difference.

CHAPTER IV

RESULTS AND DISCUSSION

1. Preparation of emblica extract and its fractions

The emblica fruit extract purchased from Specialty Natural Product Co., Ltd., SNP, was yellow-brown powder. The in-house emblica fruit extract, ESDE, was pale yellow powder with distinct odor. In this study ESDE Lot1 was prepared from 115 kg fresh emblica fruits. They were crushed and filtered then dried by spray dry method giving rise to 2.2 kg of ESDE equivalent to 1.91% yield. ESDE Lot2 was prepared from 258.3 kg fresh emblica fruits by the same method and yield 12.5 kg of ESDE equivalent to 4.84% yield.

The purpose of this study was to extract chemical components present in ESDE by various organic solvents. Different compound would be extracted by different organic solvents based on solubility of the compound or dielectric constant of the solvent. Thus, each fraction would contained different chemical entities and might show different biological activities.

However, only ESDE Lot1 was used as a raw material in this extraction study. The SNP and the ESDE were extracted with different organic solvents according to scheme 1 giving rise to five fractions, i.e. AC, EA, MN, ACS and MNS (Table 1). Most of the fractions are viscous with the color variation from yellow, light brown to dark brown (Figure 8).

Table 1 The percentage yield of emblica extract and its fractions after using solvent extraction

Fraction	% Yield		
	SNP	**ESDE Lot1**	**ESDE Lot2**
SD	N/A	1.91	4.84
AC	0.078	42.28	- [b]
MN	14.66	75.63	- [b]
EA	0.12	16.48	- [b]
ACS	0.25[a]	3.79[a]	- [b]
MNS	10.20[a]	11.99[a]	- [b]

[a] calculated from the input of EA, [b] did not process

In general, fractions obtained from ESDE gave higher percentage yield than those obtained from SNP. The MN fraction showed the highest percentage yield among fractions obtained from each sources. This observation implied that chemical entities in emblica fruit extract powder were water soluble compounds since methanol possesses high dielectric constant similar to the dielectric constant of water. Furthermore, percentage yield of each fraction obtained from ESDE were higher than percentage yield of each corresponding fraction obtained from SNP. This observation suggested that ESDE contained active ingredients at a higher concentration than SNP. This speculation was supported by the manufacturer that, during the preparation of SNP the manufacturer did add maltodextrin in order to increase the stability of emblica fruit extract.

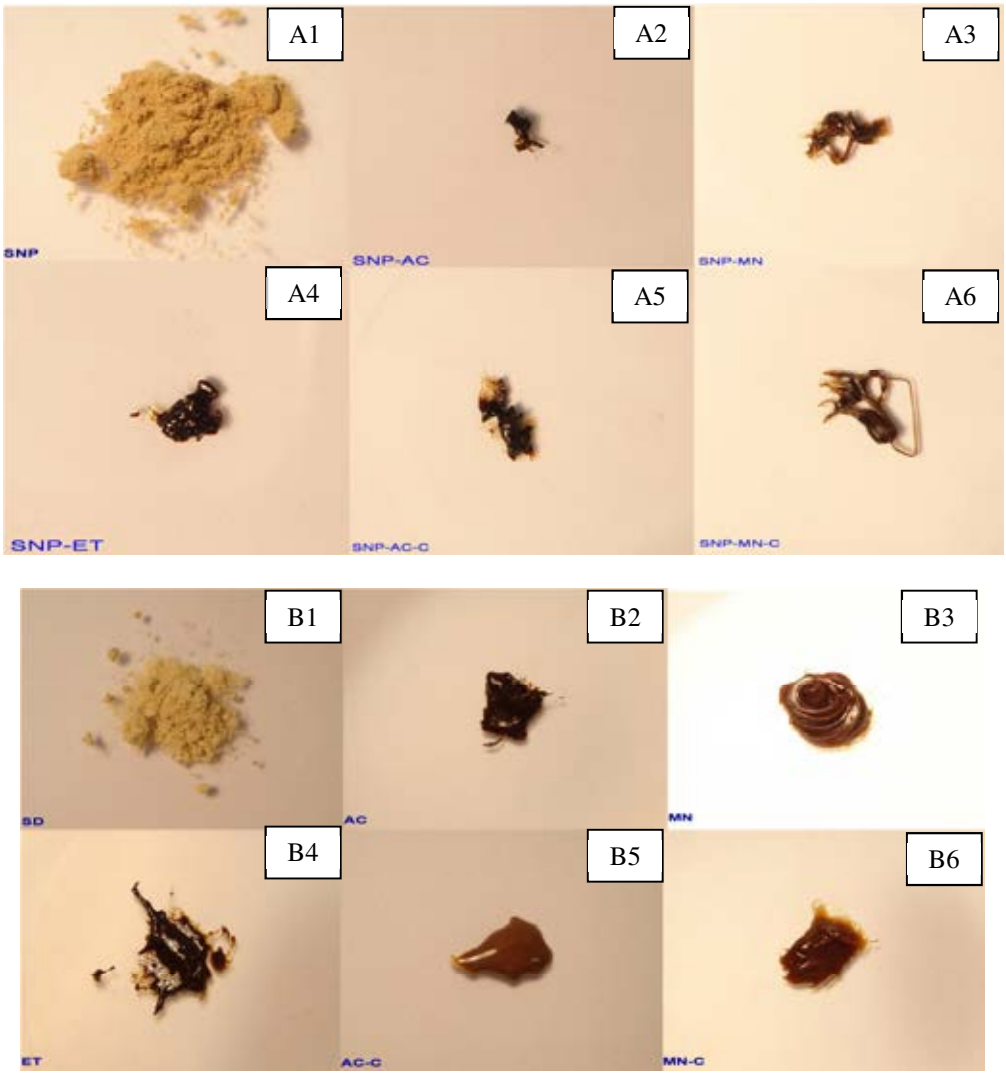


Figure 8 Appearance of SNP and its fractions; SNP (A1), AC (A2), MN (A3), EA (A4), ACS (A5) and MNS fractions (A6). Appearance of ESDE and its fractions; ESDE (B1), AC (B2), MN (B3), EA (B4), ACS (B5) and MNS fractions (B6).

2. Characterization of EFEP and their fractions

2.1 HPLC Characterization

The HPLC conditions used in this study were further modified from the HPLC conditions previously reported by อุบลทิพย์ นิมมานนิตย์ และคณะ (2550) The parameters which were modified included the porosity of the packing material of column, column's length (150, 250 mm), concentration of TFA in the mobile phase and methanol content in the mobile phase.

The HPLC method for analysis of gallic acid was validated according to ASEAN guidelines for validation of analytical procedures (Appendix B). Briefly, the method showed linearity in a concentration range of 4-32 µg/mL with typical R^2 of 0.999. Accuracy was shown in terms of percent recovery around 99%. Precision was determined as intra-day precision and inter-day precision with %RSD less than 0.48%, the LOD and LOQ were calculated to be 0.161 and 0.488 µg/mL, respectively.

In this study, gallic acid was employed as a marker since gallic acid is a polyphenolic compound known to present in emblica fruit extract. Based on the literature review, gallic acid is also a subunit of other polyphenolic compounds known to present in emblica fruit extract such as corilagin and geraniin. Furthermore, gallic acid is shown to possess antimicrobial activity (Chanwitheesuk et al., 2007). Therefore, monitoring the presence of gallic acid in EFEP and its fractions would be a simple and effective way to keep close watch over the emblica fruit extract and/or its fractions, and predict stability of formulated products. The gallic acid content in SNP, ESDE and their fractions were calculated and presented in Table 2.

Table 2 Gallic acid content of SNP, ESDE and their fractions

Fractions	% Gallic acid (w/w)	
	SNP	**ESDE Lot 1**
Powder	0.87	0.31
AC	14.77	8.44
MN	NA	0.93
EA	30.03	14.06
ACS	NA	6.84
MNS	NA	0.66

NA: can not determine from existing standard curve

The SNP and its fractions were separately dissolved in methanol and analyzed for its chemical composition using standard gallic acid as a marker. The separation was done on a Luna 5u C18 column using 0.3% v/v TFA: methanol (92:8, v/v) as a mobile phase. Chromatogram of standard gallic acid showed one peak with a retention time of around 4.3 minutes (Figure 9a). Chromatogram of SNP showed two major peaks at retention times of around 2 and 4.3 minutes corresponding to an unknown compound and gallic acid, respectively (Figure 9b). Chromatograms of each fraction obtained from SNP also showed that its contained gallic acid as a major compound (Figure 9c-9e). Gallic acid was better extracted by ethyl acetate and acetone than by methanol (Figure 9c, 9d, 9e). The sequential extraction process; a series of extraction starting from ethyl acetate, acetone and methanol, acetone fraction gave higher gallic acid whereas methanol gave lower amount of gallic acid (Figure 9e, 9f, 9g).

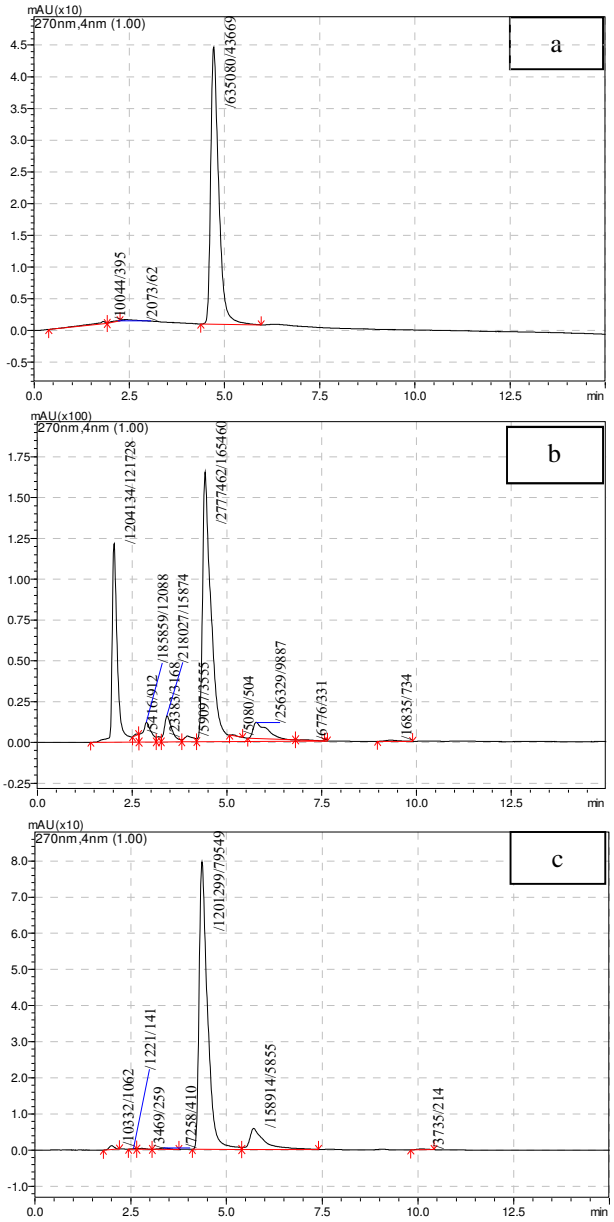


Figure 9 Chromatograms of emblica extract (SNP) and its fractions incomparision with chromatogram of standard gallic acid 10 µg/mL (a); SNP 5112 µg/mL (b), EA 111.8 µg/mL (c), AC 486 µg/mL (d), MN 40.8 µg/mL (e), ACS 676 µg/mL (f) and MNS 67.2 µg/mL (g) . HPLC conditions; HPLC: Shimadzu LC-20, Column: Luna 5u C18(2) 100A 150x4.6 mm, Mobile phase: 0.3% v/v TFA:methanol (92:8, v/v) , flow rate: 1 mL/min , injection volume: 20 µL, detection: 270 nm, column temperature: ambient, run time: 15 minute.

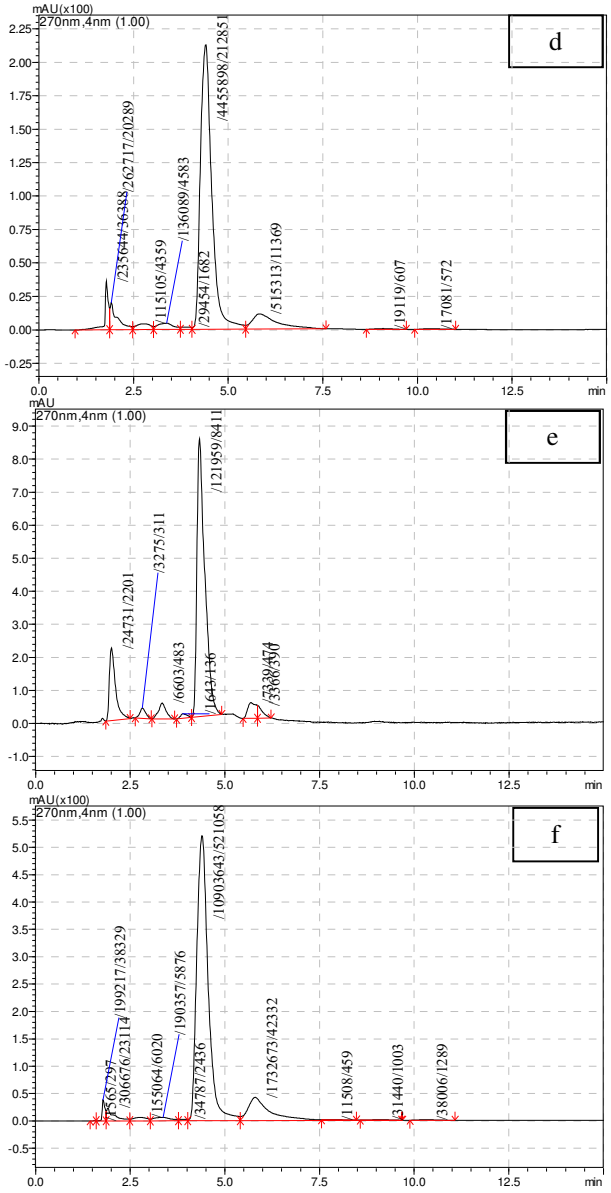


Figure 9 (cont) Chromatograms of emblica extract (SNP) and its fractions incomparision with chromatogram of standard gallic acid 10 µg/mL (a); SNP 5112 µg/mL (b), EA 111.8 µg/mL (c), AC 486 µg/mL (d), MN 40.8 µg/mL (e), ACS 676 µg/mL (f) and MNS 67.2 µg/mL (g) . HPLC conditions; HPLC: Shimadzu LC-20, Column: Luna 5u C18(2) 100A 150x4.6 mm, Mobile phase: 0.3% v/v TFA:methanol (92:8, v/v) , flow rate: 1 mL/min , injection volume: 20 µL, detection: 270 nm, column temperature: ambient, run time: 15 minute.

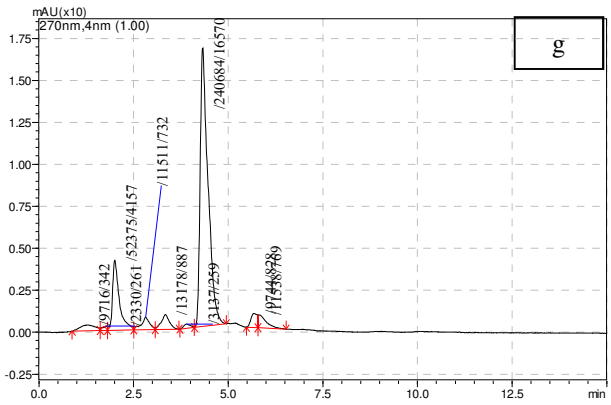


Figure 9 (cont) Chromatograms of emblica extract (SNP) and its fractions incomparision with chromatogram of standard gallic acid 10 µg/mL (a); SNP 5112 µg/mL (b), EA 111.8 µg/mL (c), AC 486 µg/mL (d), MN 40.8 µg/mL (e), ACS 676 µg/mL (f) and MNS 67.2 µg/mL (g) . HPLC conditions; HPLC: Shimadzu LC-20, Column: Luna 5u C18(2) 100A 150x4.6 mm, Mobile phase: 0.3% v/v TFA:methanol (92:8, v/v) , flow rate: 1 mL/min , injection volume: 20 µL, detection: 270 nm, column temperature: ambient, run time: 15 minute.

The ESDE and its fractions were analyzed by HPLC with different HPLC conditions. The separation was done on a Luna 5u C18 column using 0.05% v/v TFA: methanol (95:5, v/v) as a mobile phase. ESDE chromatogram showed that ESDE contained at least six major compounds at retention time around 3, 5.3, 6.7, 8.3, 9,9 and 11.9 minutes (Figure 10b). The peak with the retention time of 8.3 minutes was assigned to be gallic acid after comparison with the relative retention time of standard gallic acid (Figure 10a). Concentration of gallic acid present in MN, AC and EA fractions was increased as dielectric constant of the solvent decreased (Figure 10c, 10d, 10e). This observation was contradict to a previous result that solubility of gallic

acid in methanol> in water > in ethyl acetate (Daneshfar, Ghaziaskar and Homayoun, 2008). However, the increase in gallic acid concentration present in the fractions could result from degradation of other polyphenolic compounds during the extraction process.

Although only one out of 6 major peaks in the HPLC chromatogram could be decisively assigned, characteristics of other peaks were studied and discussed herein. The presence of compounds with retention time around 5.3 and 6.7 minutes were also depended on dielectric constant of the solvents. As dielectric constant value increased, peak area of peak with the retention time of 5.3 minutes was decreased while peak area of peak with the retention time of 6.7 minutes was increased (Figure 10b-10d). In addition, mass analysis of compounds eluted at retention time of 5.3 and 6.7 minutes revealed mass to charge ratio (m/z) of 342.9 and 444.9, respectively. Furthermore, peaks at relative retention times around 3, 9.9 and 11.9 minutes, observed in ESDE and its fractions, showed m/z of 360.9, 113.0 and 342.9, respectively. However, based on current information, these compounds could not be conclusively identified.

In this study, ESDE Lot 2 was prepared and used in the formulation of GCE. It had no major differences from ESDE Lot 1. Apart from the total phenolics content, other chemical and physical properties except were analyzed. The results showed ESDE Lot 2 was conformed with the specifications (อุบลทิพย์ นิมมานนิตย์, รศ.ดร. และคณะ, 2550, Appendix A).

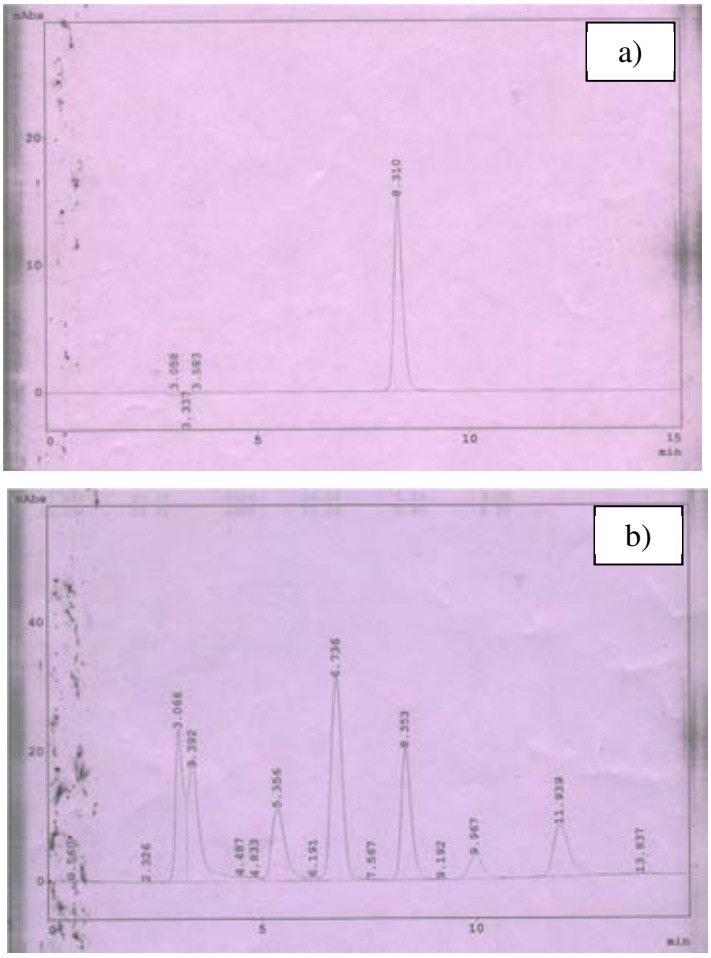


Figure 10 HPLC chromatogram of emblica extract (ESDE) and its fraction incomparison with chromatogram of standard gallic acid 4 µg/mL (a); ESDE (b), AC (c), MN (d), EA (e), ACS (f) and MNS (g) fractions concentration around 2000 µg/mL HPLC conditions; HPLC: Shimadzu LC-10, Column: Luna 5u C18, 120A 250x4.6 mm, Mobile phase: 0.05% v/v TFA:methanol (95:5, v/v) , flow rate: 1 mL/min, injection volume: 20 µL, detection: 270 nm, column temperature: 30 ^{o}C, run time: 15 minute.

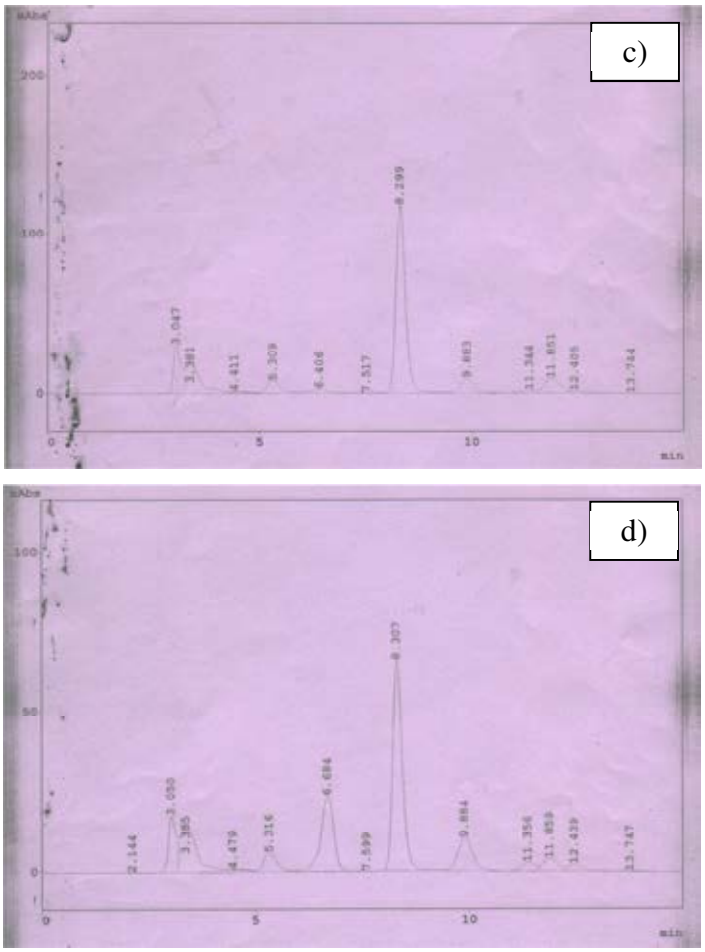


Figure 10 (cont) HPLC chromatogram of emblica extract (ESDE) and its fraction incomparison with chromatogram of standard gallic acid 4 µg/ml (a); ESDE (b), AC (c), MN (d), EA (e), ACS (f) and MNS (g) fractions concentration around 2000 µg/ml HPLC conditions; HPLC: Shimadzu LC-10, Column: Luna 5u C18, 120A 250x4.6 mm, Mobile phase: 0.05% v/v TFA:methanol (95:5, v/v) , flow rate: 1 mL/min, injection volume: 20 µL, detection: 270 nm, column temperature: 30 ^{o}C, run time: 15 minute.

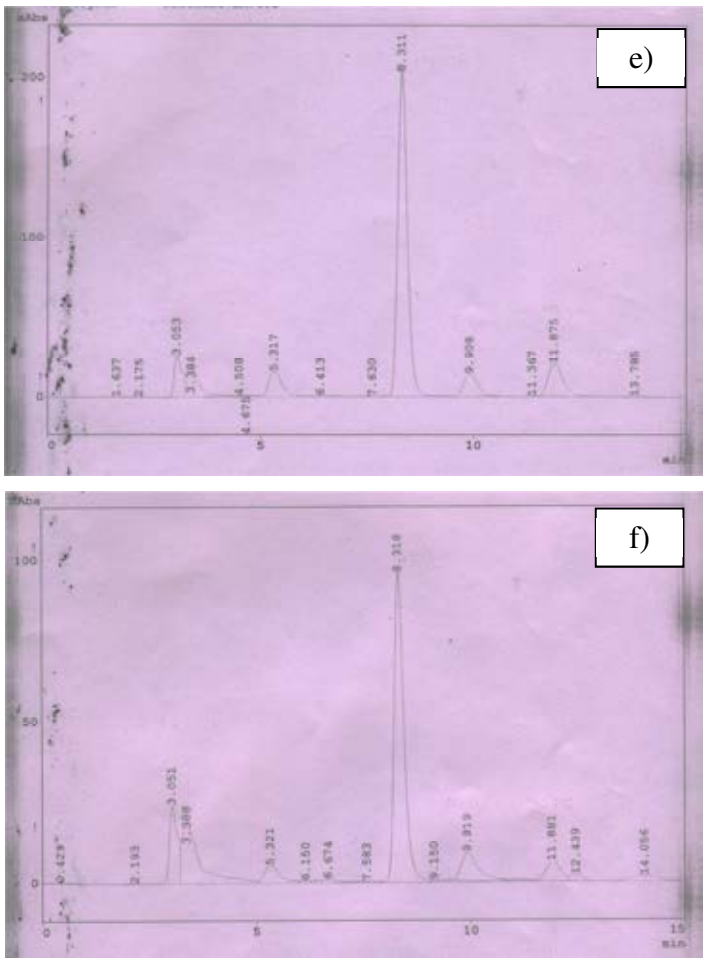


Figure 10 (cont) HPLC chromatogram of emblica extract (ESDE) and its fraction incomparison with chromatogram of standard gallic acid 4 µg/ml (a); ESDE (b), AC (c), MN (d), EA (e), ACS (f) and MNS (g) fractions concentration around 2000 µg/ml HPLC conditions; HPLC: Shimadzu LC-10, Column: Luna 5u C18, 120A 250x4.6 mm, Mobile phase: 0.05% v/v TFA:methanol (95:5, v/v) , flow rate: 1 mL/min, injection volume: 20 µL, detection: 270 nm, column temperature: 30 ^{o}C, run time: 15 minute.

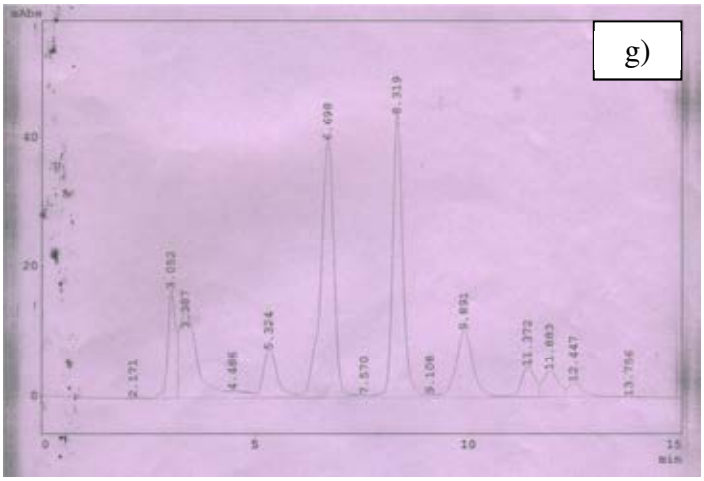


Figure 10 (cont) HPLC chromatogram of emblica extract (ESDE) and its fraction incomparison with chromatogram of standard gallic acid 4 µg/ml (a); ESDE (b), AC (c), MN (d), EA (e), ACS (f) and MNS (g) fractions concentration around 2000 µg/ml HPLC conditions; HPLC: Shimadzu LC-10, Column: Luna 5u C18, 120A 250x4.6 mm, Mobile phase: 0.05% v/v TFA:methanol (95:5, v/v) , flow rate: 1 mL/min, injection volume: 20 µL, detection: 270 nm, column temperature: 30 ^{o}C, run time: 15 minute.

2.2 Determination of total phenolic compounds

In this study, typical standard curve and its regression equation were shown in Appendix B. The content of total phenolic compounds in ESDE and its fractions were calculated from the linear regression equation and reported as % total phenolic compounds (Table 3). In case of ESDE and its fractions, EA fraction showed the highest phenolic content, follow by the AC fraction, the MN fraction, the ACS fraction, the MNS fraction and the ESDE, respectively. In general, by reducing dielectric constant of solvent from water to ethyl acetate, percent phenolic constant in

emblica extract was increased by almost 2 times. The above results suggested that polarity of polyphenolic compounds in emblica fruit were less than polarity of water and methanol.

Folin-Ciocalteau's reagent reacts with phenolic functional group. Therefore, this technique could estimate overall phenolic content present in the samples. Polyphenols are divided into water-soluble, such as catechin and epicatechin and water-insoluble compounds, such as furosin, corilagin and geraniin. In nature, polyphenolic compounds with lower polarity are present in the glycosylated form, a form with higher water solubility (Mueller-Harvey, 2001).

Table 3 Percent phenolic compounds present in SNP, ESDE and fractions

Fraction	**% phenolic compound**	
	SNP (n=1)	**ESDE Lot1 (n=3)**
EFEP	2.95	15.77 $\pm$ 0.33
MN	13.55	19.58 $\pm$ 0.15
AC	31.37	23.97 $\pm$ 0.06
EA	27.34	29.92 $\pm$ 0.21
ACS	29.62	17.96 $\pm$ 0.09
MNS	14.08	17.39 $\pm$ 0.26

Taking all together, the higher percentage yield of ESDE and its fractions related to the higher content of total phenolic compounds and the presence of other compounds including gallic acid. The ESDE and its fractions were selected for further studies.

2.3 Screening test for antimicrobial activity

2.3.1 *In vitro* antimicrobial activity of the emblica crude extract and fractions

ESDE and its fractions were screened for its antimicrobial activity using a modified cup plate method. Three bacteria were used, i.e. *S. aureus* ATCC 25923, *P. aeruginosa* ATCC 27853 and *E. coli* ATCC 25922. In this study, ESDE sample was prepared in water. Other fractions with poor water solubility were dissolved in DMSO. A preliminary result showed that ESDE fractions could not be dissolved in DMSO-aqueous solution but dissolved in 100% DMSO. Thus, pure DMSO was used as the solvent of other ESDE fractions and used as a negative control.

The ESDE showed antimicrobial activity against *S. aureus* ATCC 25923 (Table 4). Although other fractions showed the inhibition zones against the 3 bacteria, the negative control sample (100% DMSO) indicated that the observed antimicrobial activity was due to the solvent effect. Therefore, the ESDE was selected for further studies.

Moreover the ESDE was prepared to the final concentration of 25, 50 and 100 mg/mL in distilled water and re-tested against three types of bacteria using a modified cup plate method. The results showed positive on *S. aureus* but there is no inhibition zone observed for *E. coli* and *P. aeruginosa* (Table 5). Data from tables 4 and 5 can be concluded that the ESDE has a potential to inhibit the gram positive bacteria, *S. aureus* but the ESDE showed no antibacterial activity against gram negative bacteria.

There is the difference in cell wall composition between gram positive and gram negative bacteria. The cell wall of gram positive bacteria mainly consists of peptidoglycan while that of gram negative bacteria consists of thin layer of

peptidoglycan and outer membrane which are mainly consist of lipid, lipoprotein and phospholipid (Nester, Roberts and Nester, 1995).

Table 4 Antimicrobial activity of ESDE and its fractions (n=1)

Fraction	Concentration (mg/mL)	Inhibition zone (mm)		
		S.aureus **ATCC 25923**	***P.aeruginosa*** **ATCC 27853**	***E.coli*** **ATCC 25922**
ESDE [a]	25	13.5	-	-
AC [b]	50	14.5	15.8	12.5
MN [b]	50	11.5	15.8	13.5
EA [b]	50	13.8	16.3	14.2
ACS [b]	50	10.2	14.7	12.7
MNS [b]	50	10.3	16.7	12.7
DMSO	-	13.5	17.5	13.3

[a] dissolved in water, [b] dissolved in 100% DMSO

Table 5 Antimicrobial activity of ESDE against *S. aureus, E. coli* and *P. aeruginosa*

Concentration (mg/ml)	***S. aureus*** **ATCC 25923**		***E. coli*** **ATCC 25922**		***P. aeruginosa*** **ATCC 27853**	
	n1	**n2**	**n1**	**n2**	**n1**	**n2**
25	11.80	9.17	-	-	-	-
50	11.50	13.33	-	-	-	-
100	16.67	16.17	-	-	-	-

2.3.2 Antimicrobial activity of ESDE against *S. aureus*

Zone of inhibition test, using a modified disc diffusion method, was employed for further screening of antibacterial activity of ESDE. In this study, antibacterial activity of ESDE against 30 isolates of *S. aureus*; i.e. a *S. aureus* ATCC 25922 and 29 clinical isolates, was determined. Tetracycline (30 µg/disc) and Clindamycin (0.02-2 µg/disc) were used as positive controls. Discs presoaked with sterile distilled water were used as a negative control.

ESDE (10 mg/disc), tetracycline (30 µg/disc) and clindamycin (2 µg/disc) showed inhibition zone against 30 isolates of *S. aureus* with diameter in a range of 9.20 to 13.78 mm, 7.25 to 29.83, and 12.33 to 24.72 mm, respectively (Table 6). While ESDE (2 mg/disc) showed inhibition zone ranged from 6.38 to 11.04 mm. It is interesting that *S. aureus* isolates no.569 did not show inhibition zone to clindamycin with the concentration up to 2 µg/disc.

The observed inhibition zone implied that the ESDE inhibited the growth of *S. aureus*. According to CLSI (2007) and Mittal, Kirshor and Siddique (2013), if *S. aureus* ATCC 25923 was susceptible to tetracycline (30 µg/disc) or clindamycin (2

µg/disc), the observed inhibition zone should be wider than 19 mm or 21 mm, respectively. In other words, 11 clinical isolates of *S. aureus* employed in this study were resistant to tetracycline. When the tested strains were sensitive to tetracycline, inhibition zones of tetracycline were larger, with diameter size in a range of 23.77 to 29.83 mm, than that of extract, which gave clear zone with diameter in a range of 10.97 to 13.78 mm. On the contrary, when the tested isolates were resistant to tetracycline, the ESDE showed larger inhibition zone size against almost all tetracycline resistant strains (10 out of 11 strains) than tetracycline (Figure 11). The extract might be a good candidate in a treatment of infection caused by tetracycline resistant *S.aureus*.

Surprisingly, 21 clinical isolates of *S. aureus* were resistant to clindamycin. Among these isolates, 8 clinical isolates were resistant to both tetracycline and clindamycin. The result from another antimicrobial susceptibility using a cup plate method showed the comparable inhibition zone in ESDE at concentration of 2000 µg/disc and 0.02 µg/disc clindamycin (Figure 12).

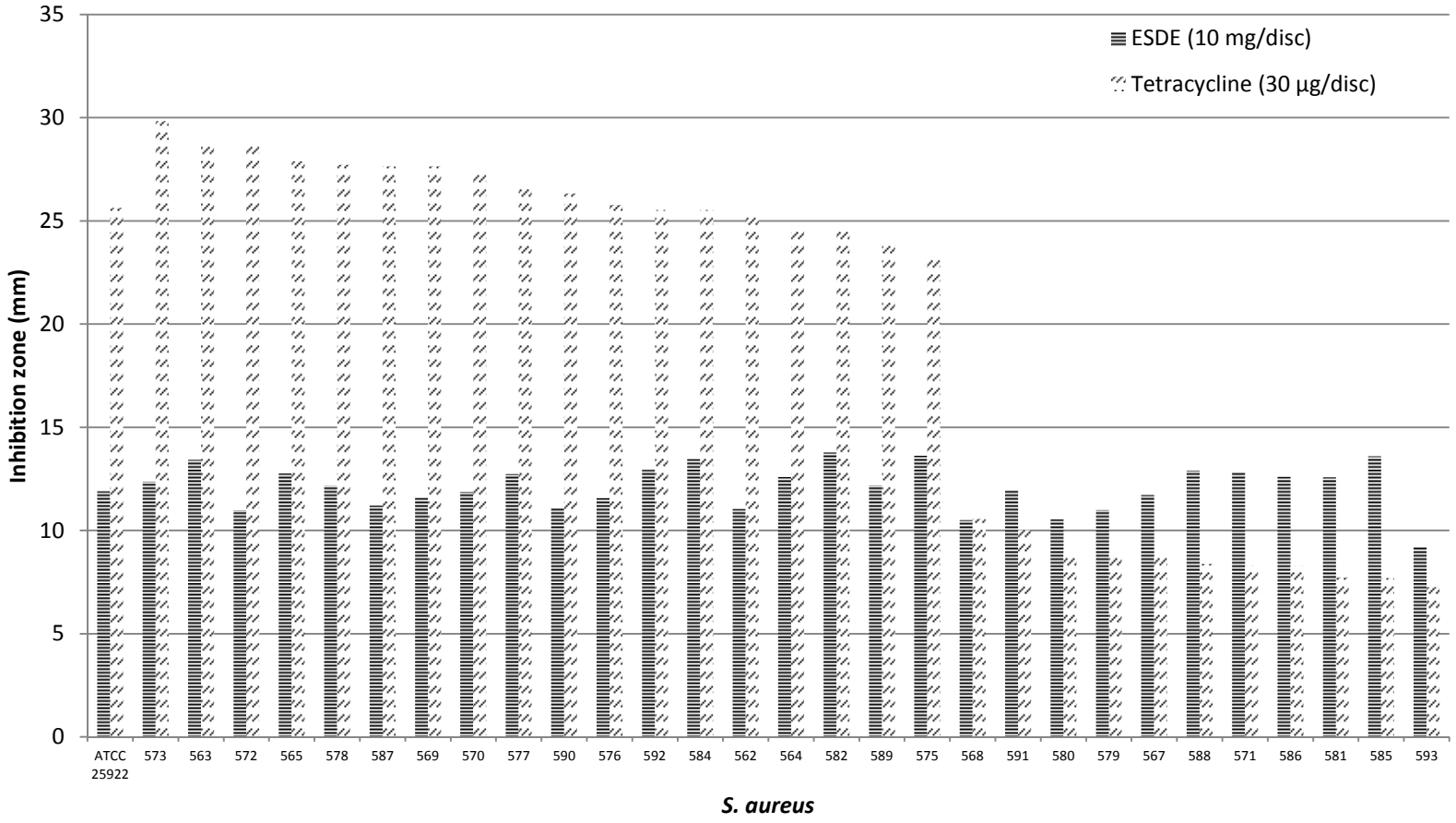


Figure 11 The antimicrobial activity of ESDE against 30 strains of *S. aureus* (tetracycline as positive control)

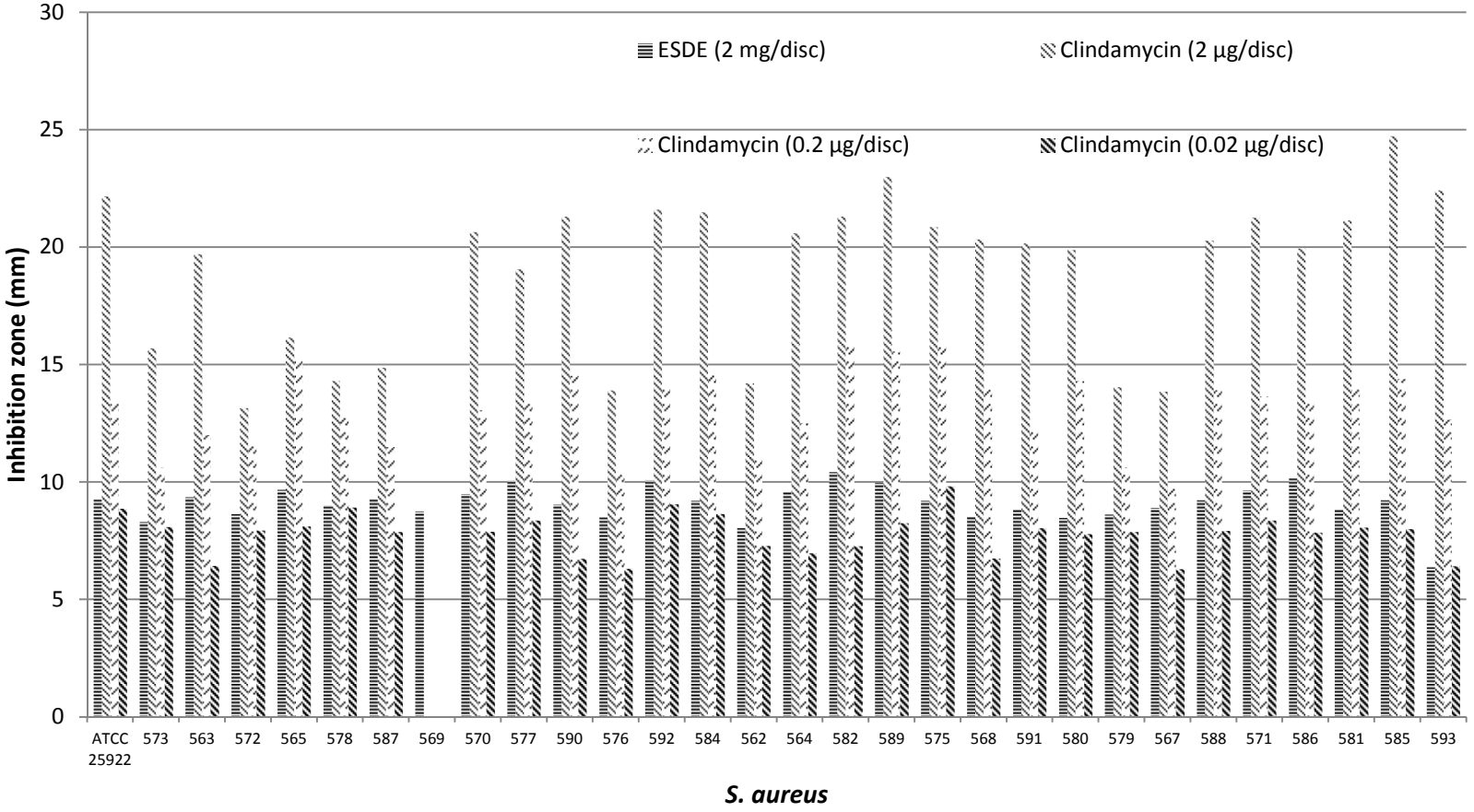


Figure 12 The antimicrobial activity of ESDE against 30 strains of *S. aureus* (clindamycin as positive control)

2.3.4 Determination of minimum inhibitory concentration (MIC)

MIC for ESDE against *S. aureus*

In this study, ESDE aqueous solution was added in to MHA agar to obtain predetermined final concentration of ESDE at 0.1, 0.5, 1, 2.5, 5 and 10 mg/mL. The 30 tested isolates of *S. aureus* were applied on each plate and then incubated at 35^{o}C for 18 hours prior to observation of bacterial growth.

At 5 mg/ml of ESDE, only *S.aureus* isolate no.593 grew visibly. At 10 mg/mL of ESDE in the solid medium, no bacterial colony was observed. Thus, MIC for ESDE against all *S. aureus* was 10 mg/mL.

By gathering all information, ESDE was chosen for further studies because it contained a higher content of total phenolic compounds than that of SNP. The polyphenols were expected to be active ingredients with antimicrobial activities (Mayachiew and Devahastin, 2008). Although EA fraction of the ESDE showed the highest content of polyphenols and gallic acid, dark brown color resin liked properties and poor water solubilities make the EA fraction obtained from ESDE unsuitable for skin applications. ESDE was shown to possess antimicrobial activity against *S. aureus*, a bacteria causing secondary infection in acne, with the MIC of 10 mg/mL. Thus, the ESDE was employed in gel preparation.

3. Development of gel containing emblica extract

3.1 Stability study of emblica extract in buffer solution

In a pH range of 3.5 to 9.0, ESDE solution is the most stable at pH 5.5 in the presence of sodium metabisulfite as antioxidant (Udomsom, Sinsuebpol and Navasinlawat, 2005). In a later study, emblica extract in water was found to be most

stable at pH around 3.0 (Chinsuwan and Siritientong, 2008) since the solution color did not change over 240 minutes at room temperature.

Snitwongs et al (2010) reported the stability study of ESDE in 0.15 M citrate buffer pH 3, 0.15 M phosphate buffer pH 3, water adjusted the pH value to 3 with 1 N HCl or water in the presence of 1% sodium metabisulfite and 0.1% sodium benzoate. pH value of each sample were monitored throughout 1 month. Mean pH values of ESDE in citrate buffer, phosphate buffer, water pH 3 and water were 3.23 $\pm$ 0.08, 3.58 $\pm$ 0.12, 3.44 $\pm$ 0.18 and 3.65 $\pm$ 0.13, respectively. Over one month storage at 30 ^{o}C, color of all samples was changed from yellow to pale yellow with the white matter sediments. Percentage of gallic acid increased (Figure 13).

Further analysis of the data, peak area and time profile of six peaks detected at 270 nm four solvents were compared (Figure 14, 15). Gallic acid was used as the external standard in every time point of sampling. The amount of peak no.1 and peak no.4 (gallic acid) increased while peak no.2, 5 and 6 decreased. Only peak no.3 did not change over the time period. Interestingly, the peak no.6 was dramatically decreased within a week. It is believed that responsible for losing of antimicrobial activity of ESDE (Figure 15). ESDE solution gave inhibition zone against five strains of *S. aureus*. When the concentration of ESDE increased from 4% to 7% w/w, the inhibition zone was wider. The observed inhibition zone was decreased when ESDE solution was stored longer time. A preservative and an antioxidant were added into ESDE solutions in order to improve its stability.

Sodium benzoate was used as a preservative since sugars in ESDE could promote growth of bacteria and/or mold. Polyphenols are known to undergo oxidation; sodium metabisulfite was shown to be a better antioxidant in preventing of

polyphenols oxidation than vitamin C and E (Udomsom et al., 2005). In addition, both compounds well performed in acidic conditions (Rowe et al., 2006). An increase in gallic acid concentration overtime indicated that polyphenols in the ESDE degraded and gave rise to gallic acid, the smallest polyphenolic structure.

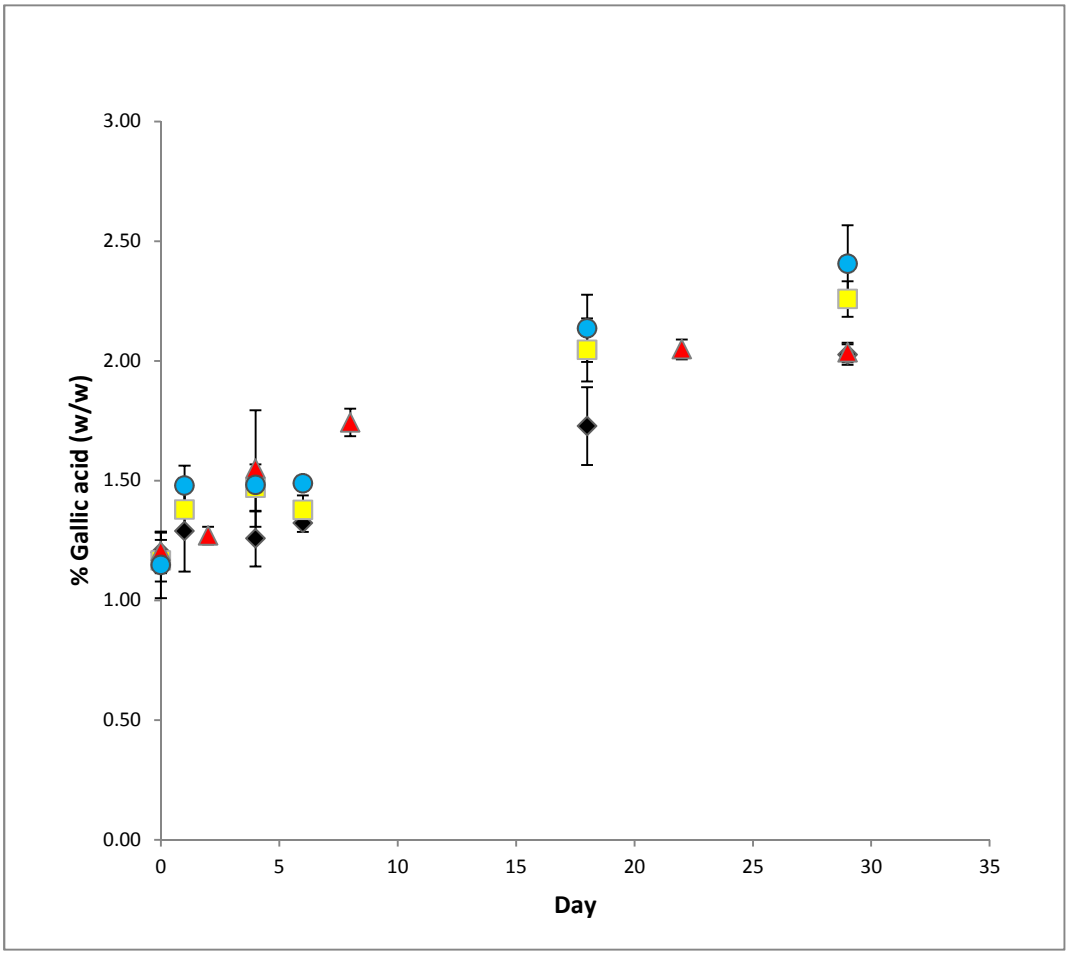


Figure 13 Percentage gallic and time profile of ESDE in four solvents; citrate buffer pH 3 (◆), phosphate buffer pH 3 (■), water adjusted pH 3 with 1N HCl (▲), water (●) (n=3)

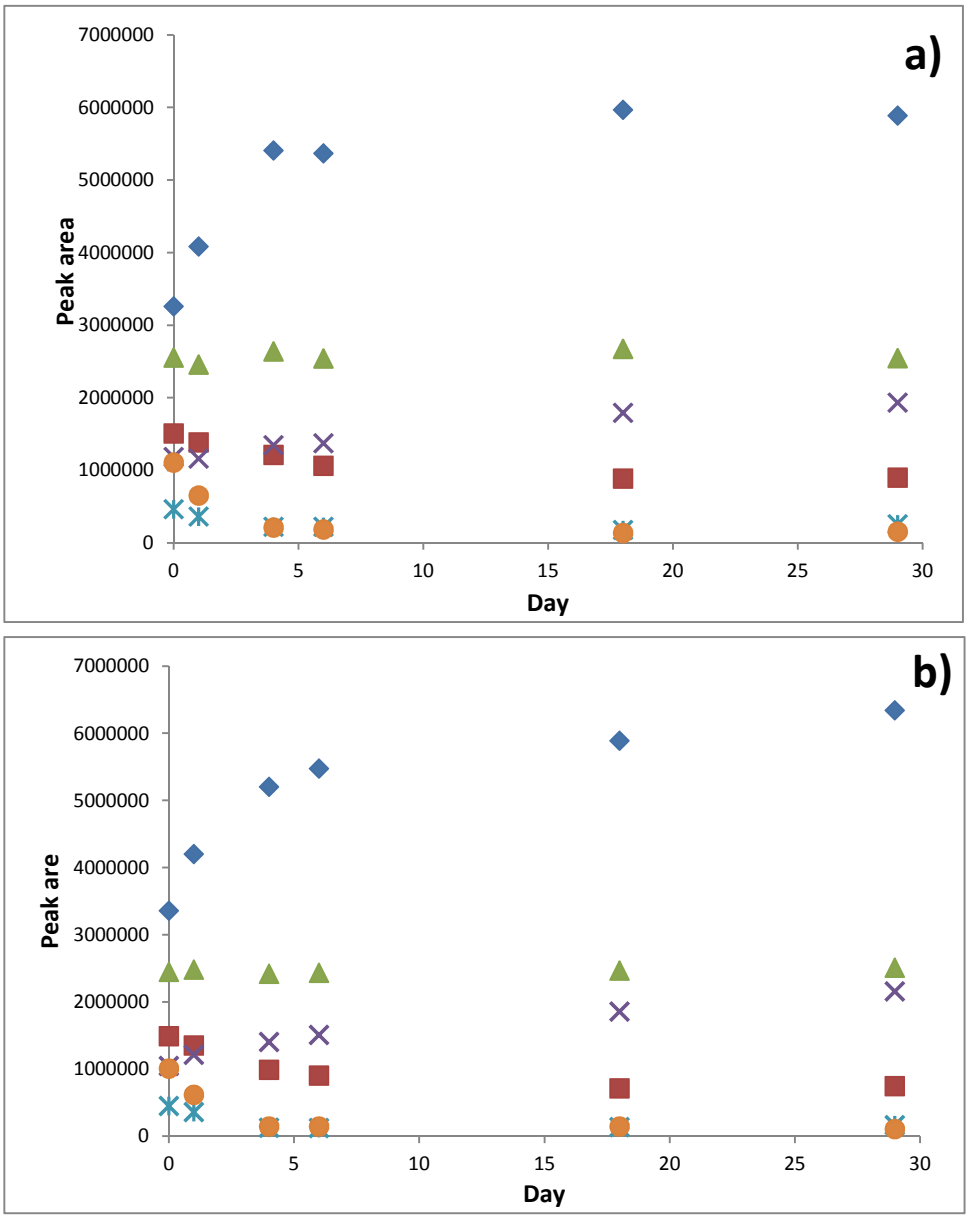


Figure 14 Peak area and time profile of six peaks detected at 270 nm; a) ESDE in citrate buffer pH 3, b) ESDE in phosphate buffer pH 3, c) ESDE in water adjusted pH 3 with 1N HCl, d) ESDE in water; peak no.1 (◆), peak no.2 (■), peak no.3 (▲), gallic acid (✕), peak no.5 (∗) and peak no.6 (●)

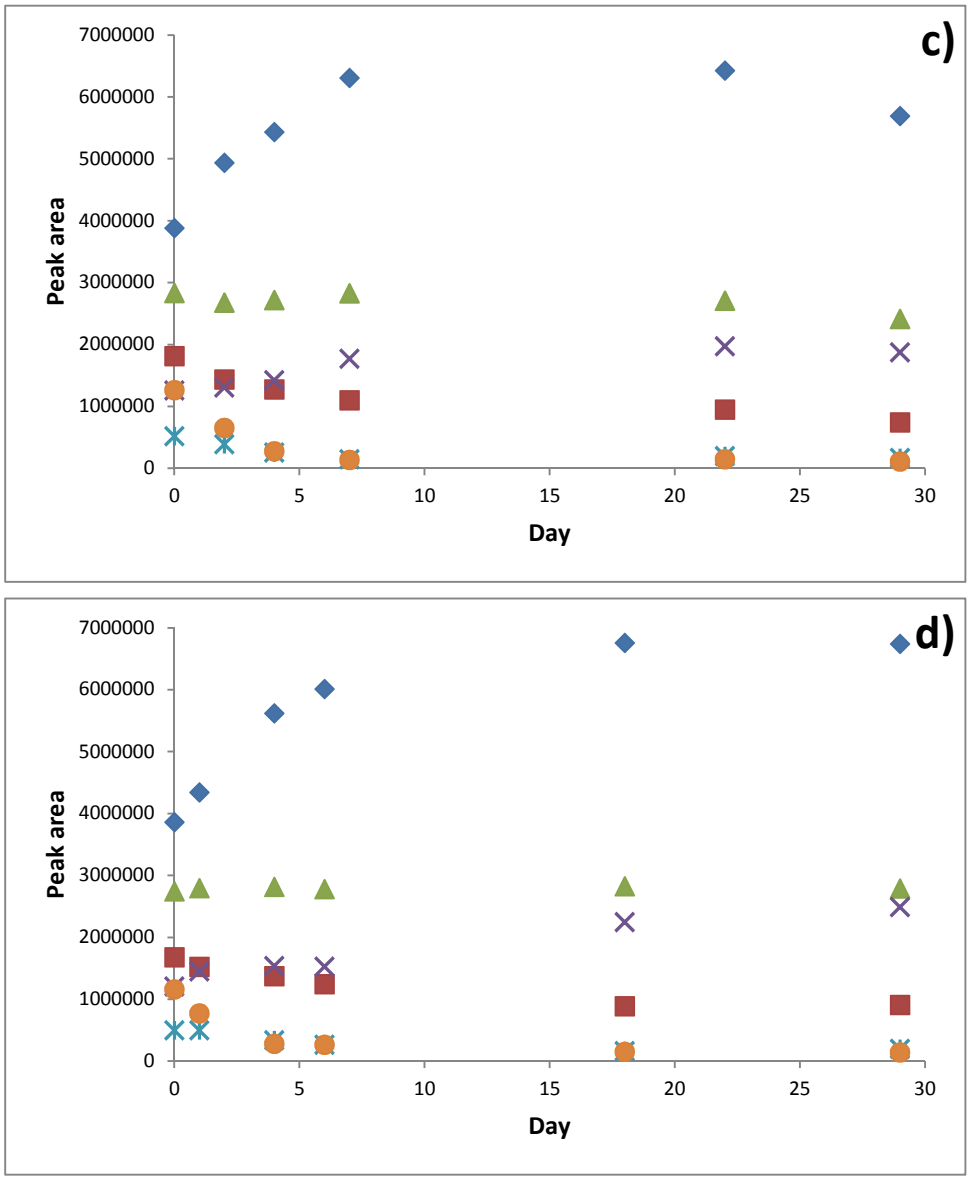


Figure 14 (cont) Peak area and time profile of six peaks detected at 270 nm; a) ESDE in citrate buffer pH 3, b) ESDE in phosphate buffer pH 3, c) ESDE in water adjusted pH 3 with 1N HCl, d) ESDE in water; peak no.1 (◆), peak no.2 (■), peak no.3 (▲), gallic acid (✕), peak no.5 (∗) and peak no.6 (●)

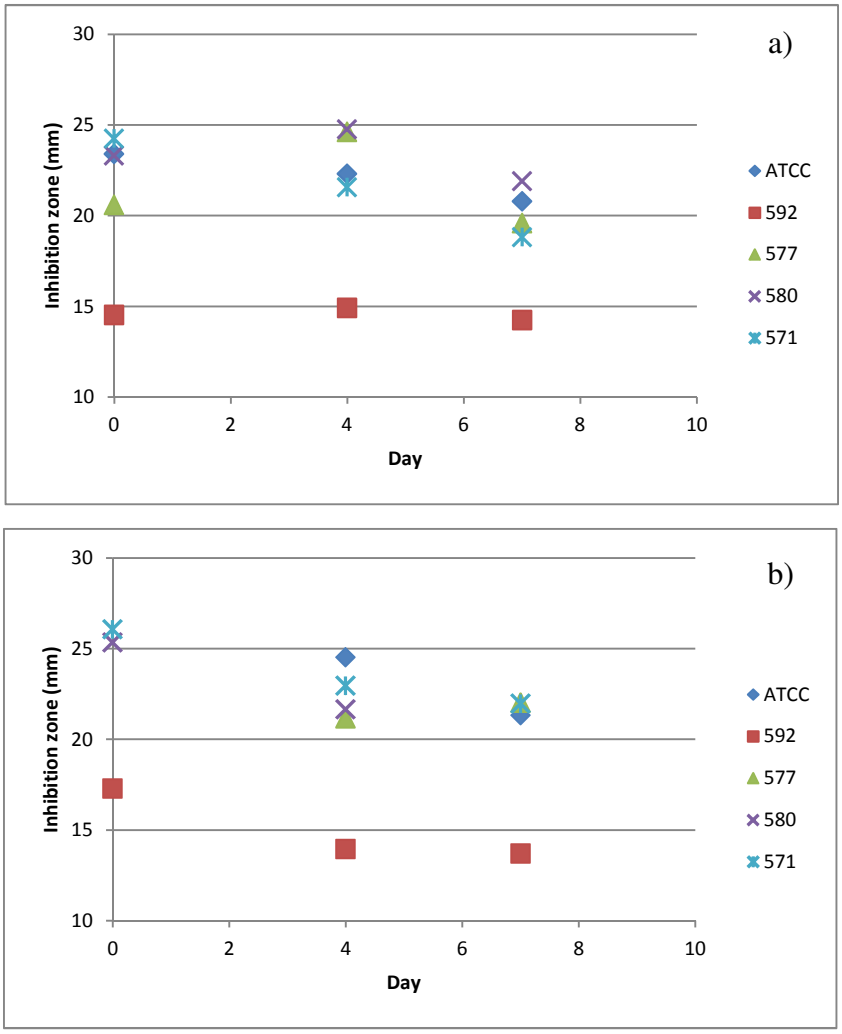


Figure 15 The inhibition zone of ESDE in 1% sodium metabisulfite solution a) 4% ESDE solution b) 7% ESDE solution

3.2 Formulation of gel containing ESDE

3.2.1 Determination of suitable ESDE concentration in the preparation

At day 0, 7, 14, 21 and 28, the ESDE solution at 4, 7, 10, 15 and 20% w/w were tested for its antimicrobial activity against 4 isolates of *S. aureus* according to the zone inhibition test. Among these four isolates, *S. aureus* ATCC 25923 and the clinical isolate number 592 were representatives of isolates that were sensitive to TC,

while the clinical isolate number 571 and 580 were resistant to TC. The inhibition zones were measured and plotted against the ESDE concentration (Figure 16). In all cases, the diameters of inhibition zone were increased as the ESDE concentration increased and reached a maximum value at ESDE concentration around 10% w/w. Thus, the ESDE concentration in the formulation was chosen to be at 10% w/w.

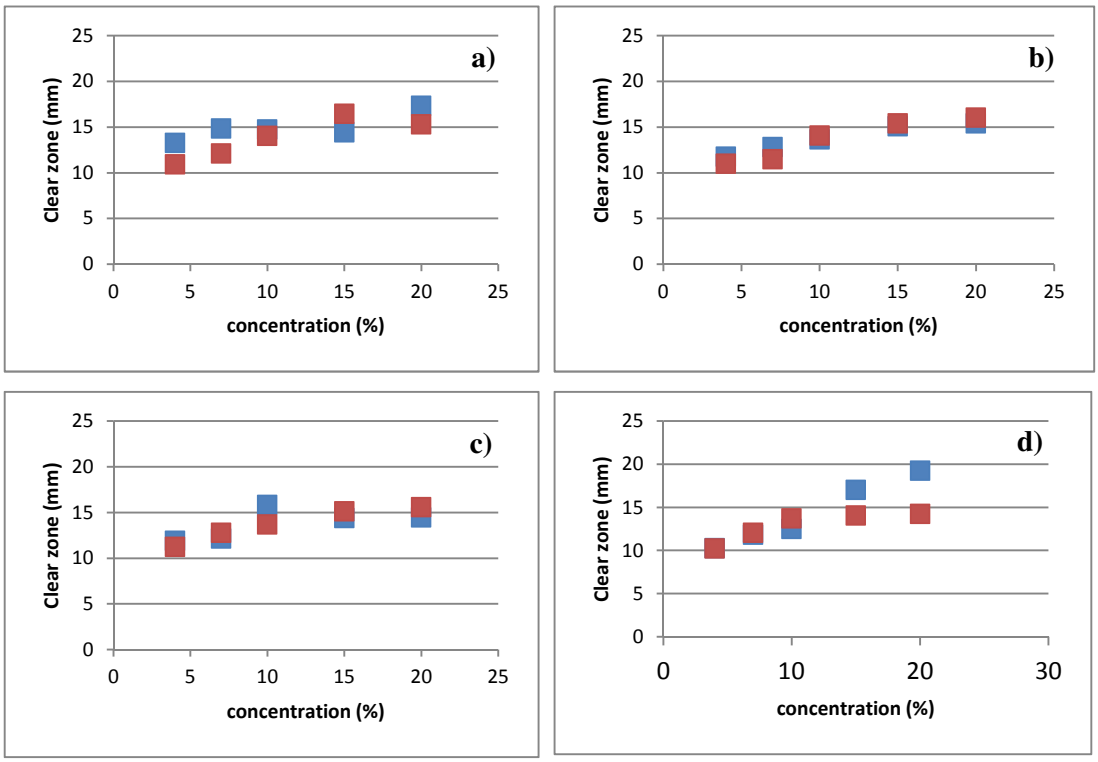


Figure 16 Inhibition zone of freshly prepared ESDE solutions at 4, 7, 10, 15 and 20% (w/w) against *S. aureus* ATCC 25923(a), clinical isolate number 592(b), 571(c) and 580(d) (n=2)

Although MIC of a compound is determined, the loading concentration in a preparation is general higher than the MIC value in order to ensure antibacterial activity of the preparation (Lemmen et al., 1993). In this research, MIC of ESDE

against 30 strains of *S. aureus* was determined to be 10 mg/mL, the loading concentration used in the preparation was around 10 times higher than its MIC value.

3.2.2 Preparation of gel containing ESDE (GCE)

The freshly prepared gel containing ESDE was translucent gel with yellow color pH 3.24 (Figure 17). The blank gel was a clear gel pH 3.59. The pH value of GCE was lower than that of the blank gel because ESDE contains acidic compounds by nature. The pH of GCE did not adjust to be equal to gel blank because the higher pH, the more degradation of polyphenolics (Chinsuwan and Siritientong, 2008). In contrast the pH of gel blank did not adjust to be equal to GCE because it was not design for the efficacy test and the physical property could be different from the prototype.

Over one month storage at 30 ^{o}C appearance of the blank gel did not change (Figure 18). However color of GCE was turned to brown color within 1 week and the color of GCE was changed to dark brown color over 1 month (Figure 16). pH values of GCE and the blank gel were quite constant. The mean pH values were 3.24 $\pm$ 0.06 and 3.59 $\pm$ 0.05, respectively (Figure 19). The viscosity of both gels was illustrated in Figure 20. The viscosity of GCE was constant throughout 1 month while that of the gel blank decreased dramatically. There was a discrepancy of the viscosity value of gel blank during the decrement of viscosity of gel blank but the inconsistent was unclear.

The decrease in viscosity of the blank gel was speculated to be due to an increase of sol-gel transition temperature of poloxamer 407 in the presence of other ingredients rather than degradation of poloxamer 407. Drugs and additives such as

diclofenac, propylene glycol, NaCl and NaH_2PO_4 were reported to greatly modify sol-gel transition temperature both by increasing and decreasing the sol-gel transition temperature (Dumortier et al., 2006). If a compound interfere micellization and dehydration of the propylene oxide block, an increase in sol-gel transition temperature will be observed. Poloxamer 407 is stable in both acidic and alkaline conditions but undergoes oxidation. Therefore, butylated hydroxytoluene (BHT) is added in commercially available poloxamer 407 (BASF, 2007). In this formulation, sodium metabisulfite, an antioxidant with the same antioxidation mechanism as that of BHT was employed. Thus, degradation of poloxamer 407 by oxidation leading to decreasing in viscosity was unlikely. However the slight decrement of viscosity in GCE remains unclear.

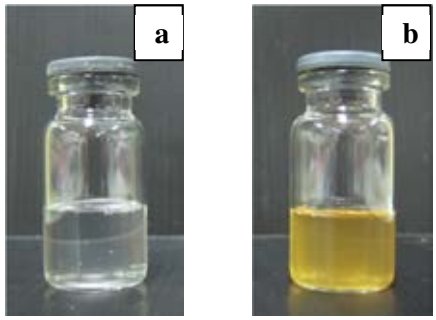


Figure 17 Gel appearance after freshly prepared, **a**) blank gel **b**) GCE

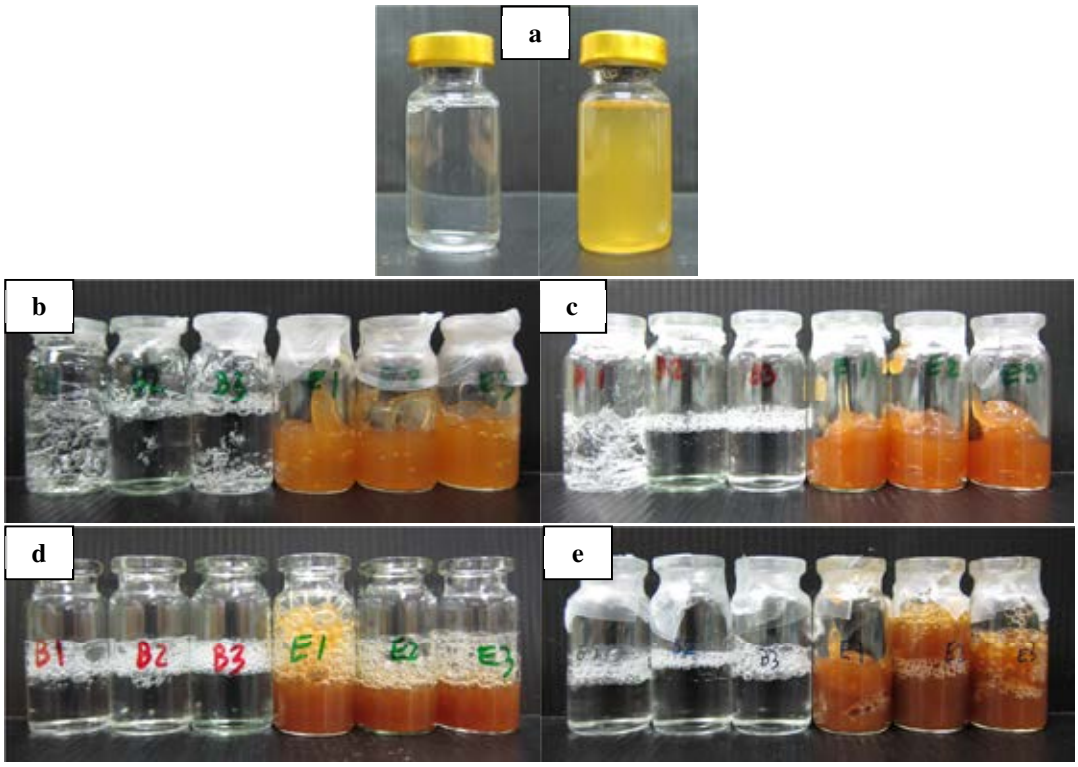


Figure 18 The color change of gel blank and GCE over 1 month storage in a 5 mL HDPE tube at 30 ^{o}C; day 0 (a), day 7 (b), day 14 (c), day 21 (d) and day 28 (e); B1-B3 were gel blanks; E1-E3 were GCE. (n=3)

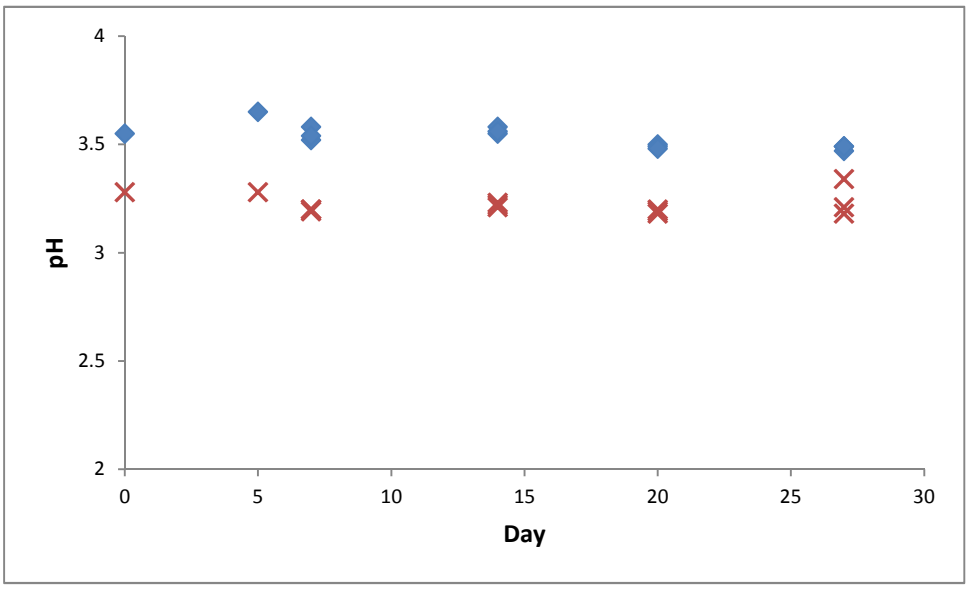


Figure 19 The pH of GCE (✕) and gel blank (◆) over 1 month storage at 30 °C (n=1 at day 0, 5, n=3 at day 7, 14, 21 and 28)

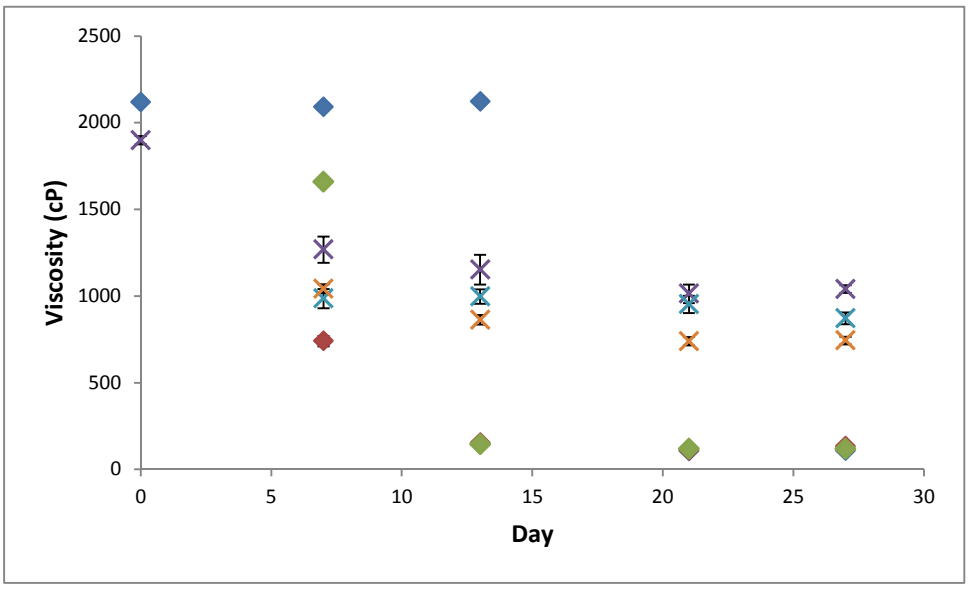


Figure 20 The viscosity of GCE (✕) and blank gel (◆) over 1 month storage at 30 °C (n=1 at day 0, n=3 at day 7, 14, 21 and 27)

Chemical stability of GCE was determined by HPLC method at day 0, 5, 7, 14, 20 and 27. Gallic acid content in GCE increased after one month storage (Figure 21). When compare the peak area time profile of GCE (Figure 22) with that of ESDE in citrate buffer pH 3 (Figure 14a), the changes of six peaks showed the same trend. Peak area of peak no.6 decreased dramatically within a week.

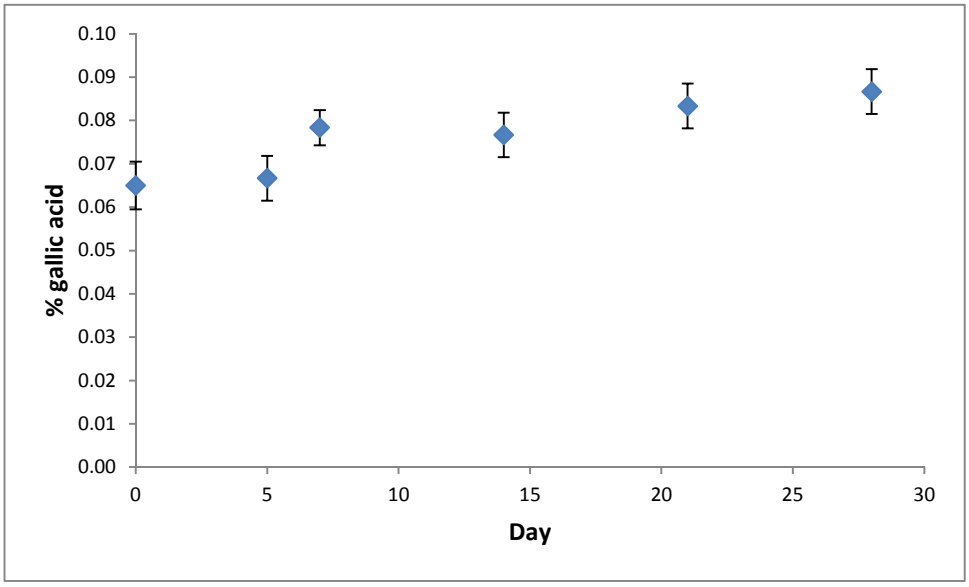


Figure 21 Gallic acid content (%, w/w) in GCE over 1 month storage at 30 ^{o}C (n=3)

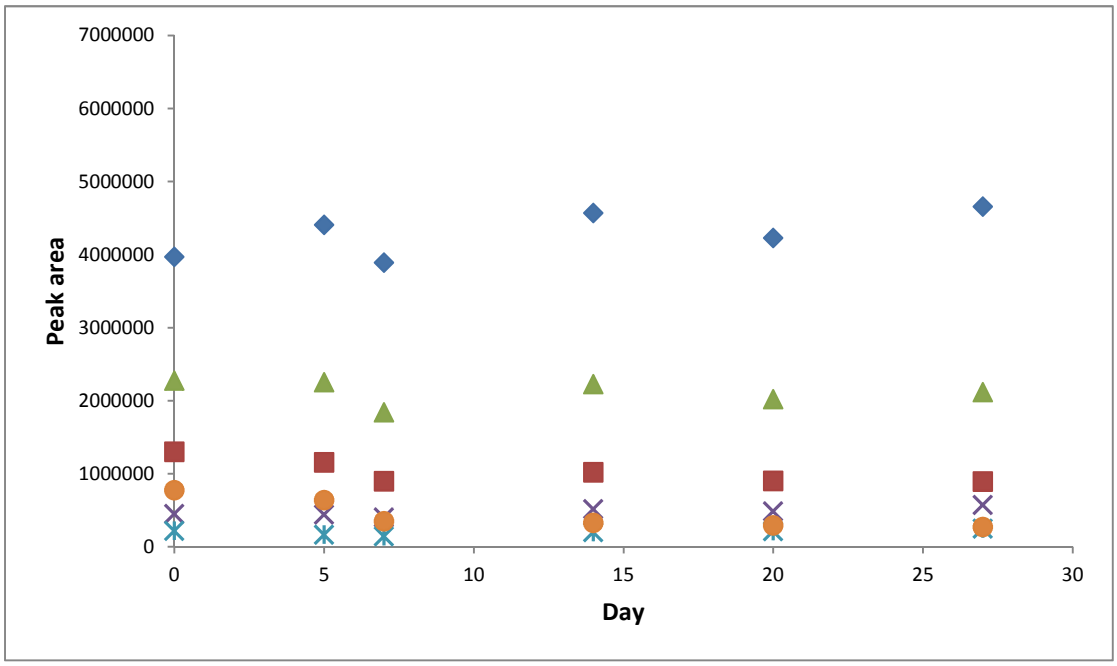


Figure 22 Peak area time profile of 6 peaks detected at 270 nm of GCE over 1 month storage at 30 ^{o}C; peak no.1 (◆), peak no.2 (■), peak no.3 (▲), gallic acid (×), peak no.5 (∗) and peak no.6 (●)

The modified cup plate method was employed in order to investigate the antimicrobial activity of GCE against *S. aureus* over 1 month period. Blank gel and 1% commercial clindamycin gel were used as negative and positive control, respectively. At day 0, diameters of the inhibition zones of 1% clindamycin, gel blank and GCE were 24, 13 and 15 mm, respectively (Figure 23, 24).

The inhibition zone of 1% clindamycin gel was quite constant. The mean inhibition zone of 1% clindamycin gel at day 0 was comparable to that of day 28 with 4.4% decrement (Figure 21). While the inhibition zones of gel blank and GCE were decreased over 1 month. The mean inhibition zone of gel blank and GCE at day 28 were decreased from day 0 by 20.8 and 15.3%, respectively.

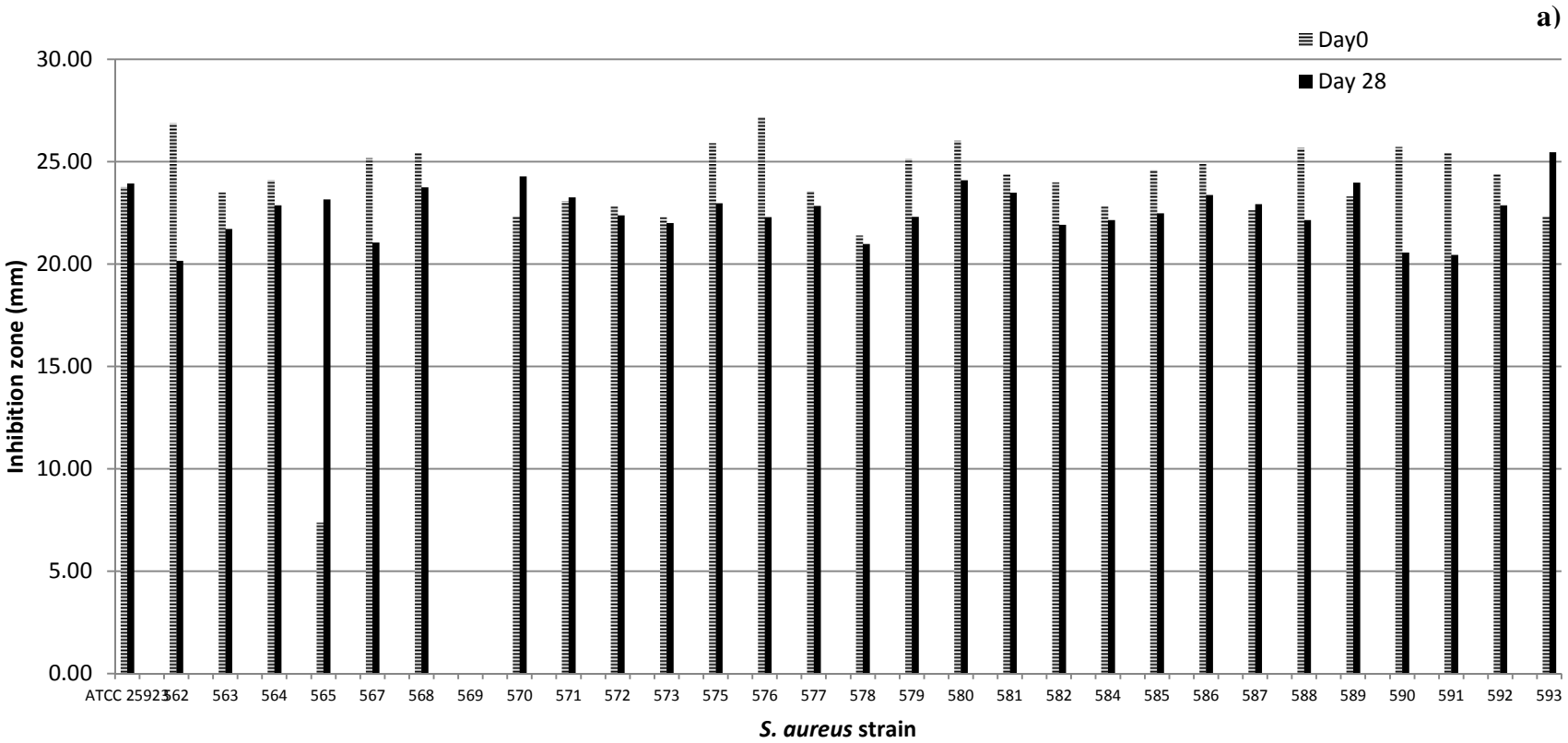


Figure 23 The inhibition zone gel sample stored at 30 ^{o}C for 1 month against 30 strains of *S. aureus*; 1% clindamycin gel (a), GCE (b), gel blank (c) (n=3)

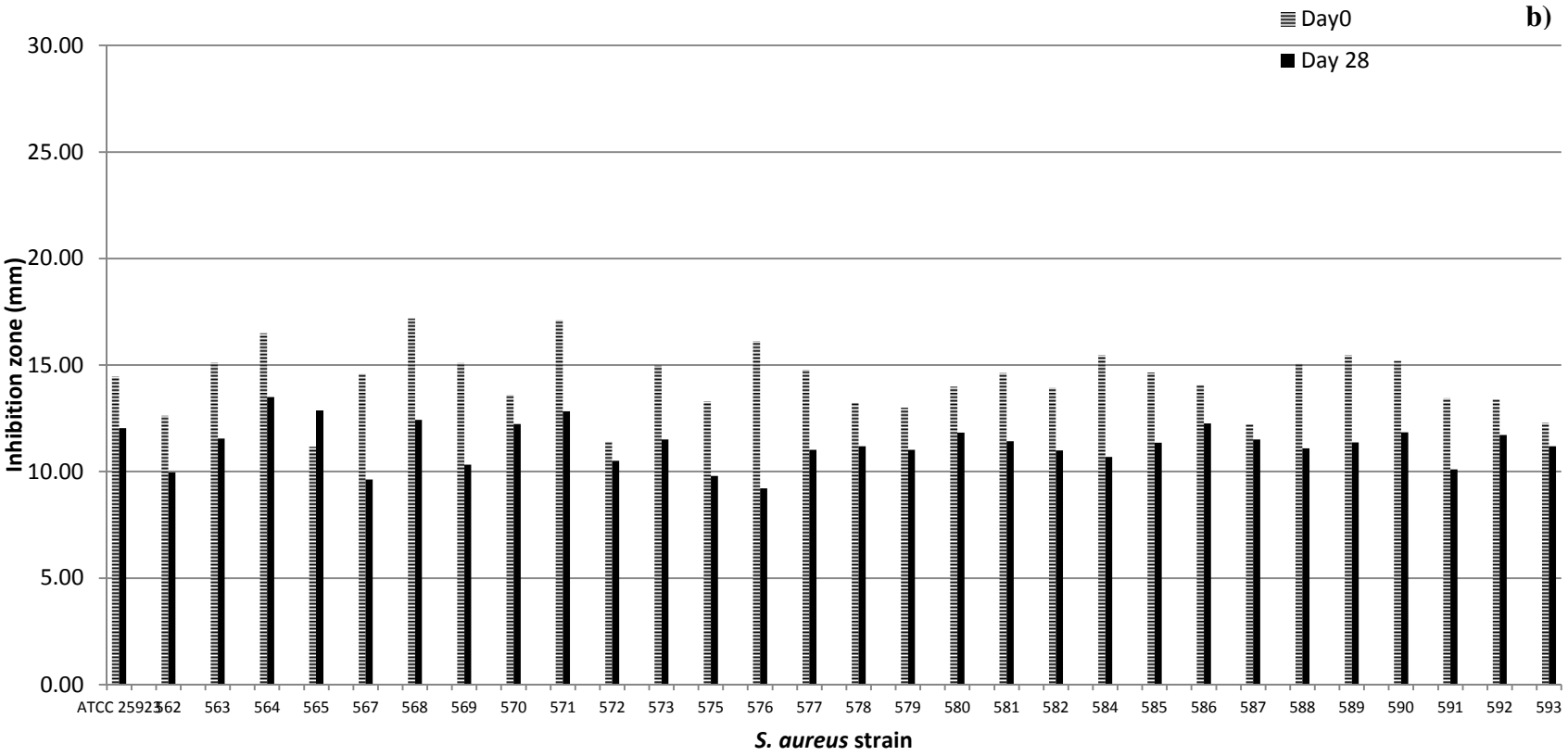


Figure 23 (cont) The inhibition zone gel sample stored at 30 ^{o}C for 1 month against 30 strains of *S. aureus*; 1% clindamycin gel (a), GCE (b), gel blank (c) (n=3)

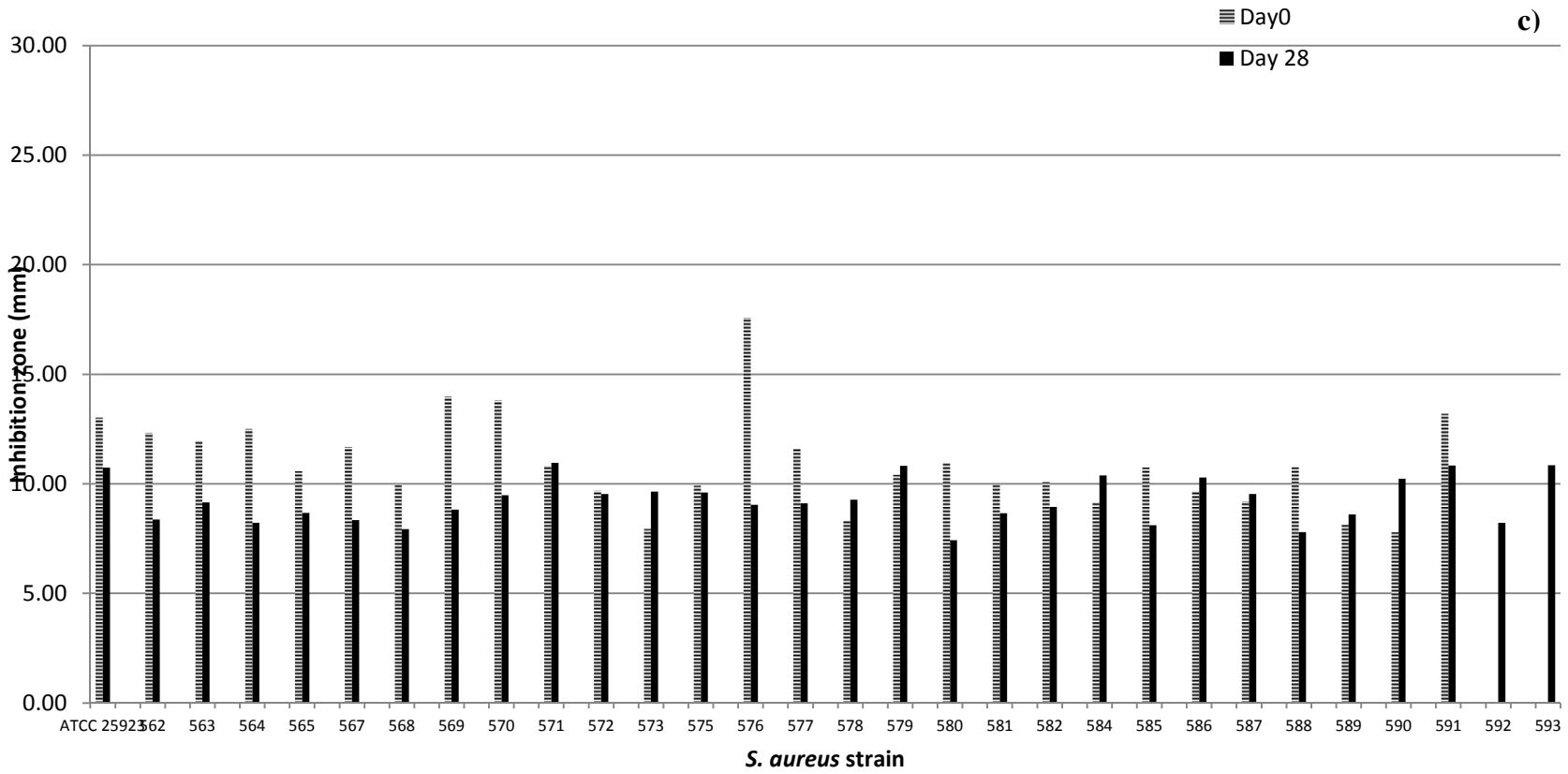


Figure 23 (cont) The inhibition zone gel sample stored at 30 ^{o}C for 1 month against 30 strains of *S. aureus*; 1% clindamycin gel (a), GCE (b), gel blank (c) (n=3)

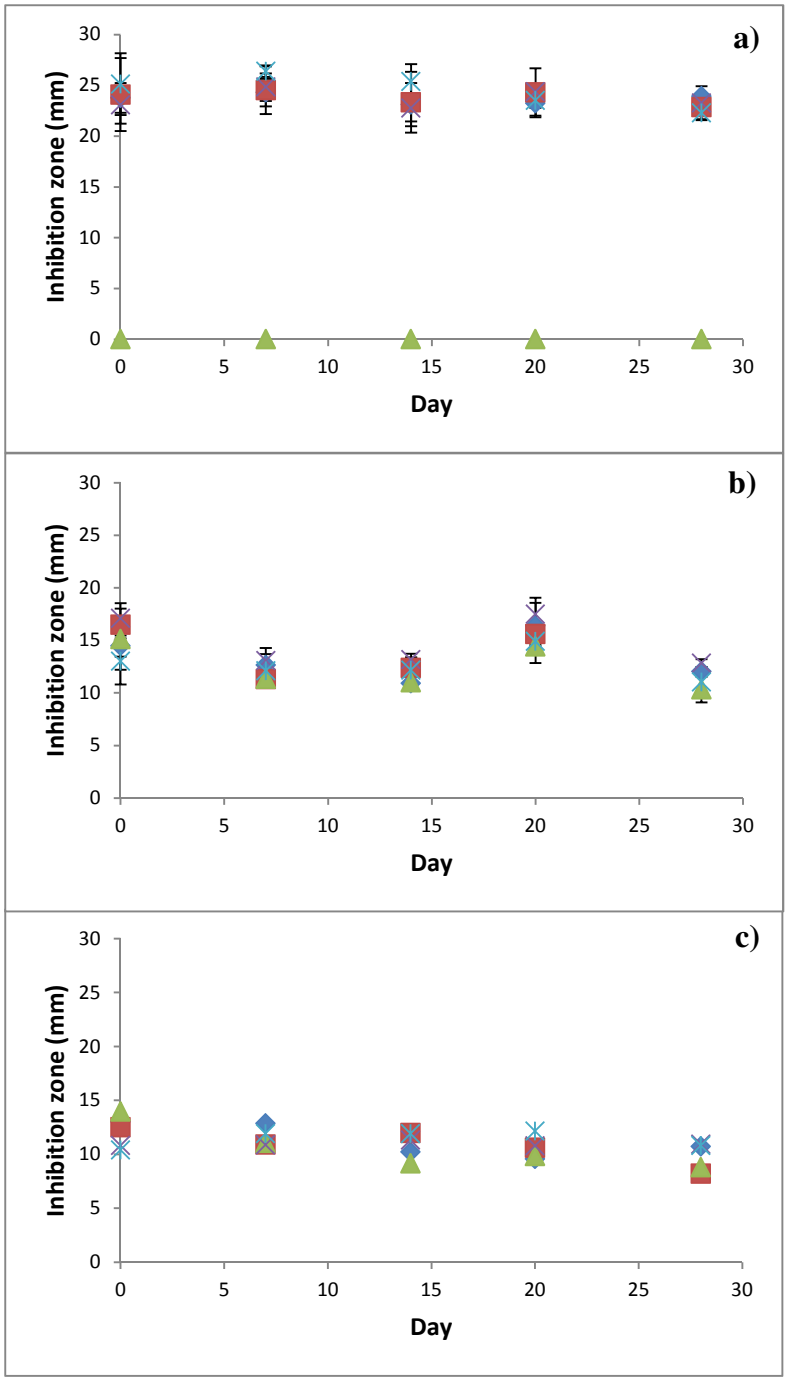


Figure 24 The inhibition zone gel sample stored at 30 °C for 1 month against 5 strains of *S. aureus*; 1% clindamycin gel (a), GCE (b), gel blank (c); *S. aureus* ATCC 25923 (◆), *S. aureus* isolates no.564 (■), 569 (▲), 571 (×) and 579 (∗) (n=3)

In general, inhibition zones observed from 1% clindamycin gel were wider than inhibition zone observed from 2 µg clindamycin disc in section 2.3.2 because the amount of clindamycin loaded in this test was 3 mg/cup. This study also confirmed the previous result in section 2.3.2 that the clinical isolate number 569 was resistant to clindamycin since the inhibition zone was not observed.

The observed inhibition zone of BLA is due to the presence of sodium benzoate and sodium metabisulfite. The pH was stable during 1 month period. The inhibition zone at day 28 did not much change from that of day 0. So it could be concluded that both chemicals did not degrade during 1 month period.

The inhibition zone of GCE at day 28 decreased from day 0. It could be the degradation of the chemical constituent(s) such as phenolic compounds that responsible for the antimicrobial activity.

GCE showed the significant change of gel color within 1 week despite the viscosity did not change. The chemical study showed the peak no.6 decreased dramatically within 1 week this corresponding to the obvious decrease of antimicrobial activity. Therefore, if GCE is packaged in a 5 mL HDPE tube and stored at 30 ^{o}C, it could be effective within a week.

4. Efficacy testing of emblica gel

Twenty-five patients, 9 men and 16 women in an age range of 18-37 years old, were recruited in this study. Thirteen patients were given the clindamycin gel and 12 patients were given GCE. Demographic data from both groups showed no significant differences from each other (Table 7). The patients were asked to visit at the beginning and at the end of each week. Number of acne, papules and pustules, and

adverse effects such as erythema, scaling, dryness, pruritus, stinging or burning were assessed. Due to the color change of GCE and the preliminary stability study on antimicrobial activity of GCE, the patients from both groups were given a new lot of gel every week. Every batch of GCE, total 6 batches, distributed to the patient has been determined its quality parameter (Table 8). After three week of study passed, a patient receiving GCE asked for termination because she did not feel comfortable to continue. Therefore, data of this volunteer were not included in the data analysis.

Table 7 Demographic data of patients recruited in the study

	Clindamycin gel	**GCE**
Enrolled	13	12
Sex (male/female)	3/10	6/6
Age (mean/range)	26.5/(20-36)	24/2/(18-37)
Discontinued		
Drop-off	0	1
Baseline values		
Number of acne (mean ± SD)		
Comedone	13.62 ± 11.50	13.08 ± 15.87
Papule	10.69 ± 2.46	11.75 ± 4.81
Pustule	1.46 ± 1.33	1.25 ± 1.71
Nodulocyst	0.38 ± 0.51	0.83 ± 1.47
GAAS (mean ± SD)	3.31 ± 0.63	3.42 ± 0.51

Both treatment groups showed percentage of acne reduction (Figure 25). The average percentage of acne reduction of patients in the clindamycin gel group and GCE were in a range of 41.4-61.2 and 33.5-56.2%, respectively. In comparison of the acne reduction of individual patient, student paired t-test of individual patient showed significant acne reduction (t_0 vs t_4). To compare percentage of acne reduction between two groups, data showed to have normal distribution with equal variance. Therefore, t-test, parametric statistics, was employed. The result showed at the end of each week, the average acne reduction between clindamycin gel and GCE are not different ($p>0.05$, Appendix D). The observed reduction in acne number could be from the anti-inflammatory effect of emblica extract (Muthuraman, Sood and Singla, 2010).

Table 8 Quality control of GCE

Lot no.	Date	% Yield	pH gel	pH buffer	%Gallic acid
001	5/6/2012	94.6	3.32	3.00	0.074
002	12/6/2012	94.5	Nd	3.01	0.072
003	19/6/2012	95.1	Nd	3.03	0.068
004	26/6/2012	94.7	3.12	3.00	0.067
005	3/7/2012	94.1	3.25	3.00	0.071
006	10/7/2012	94.7	3.3	3.01	0.068
	mean	94.62	3.25	3.01	0.070
	SD	0.33	0.09	0.01	0.003

Nd: not determine

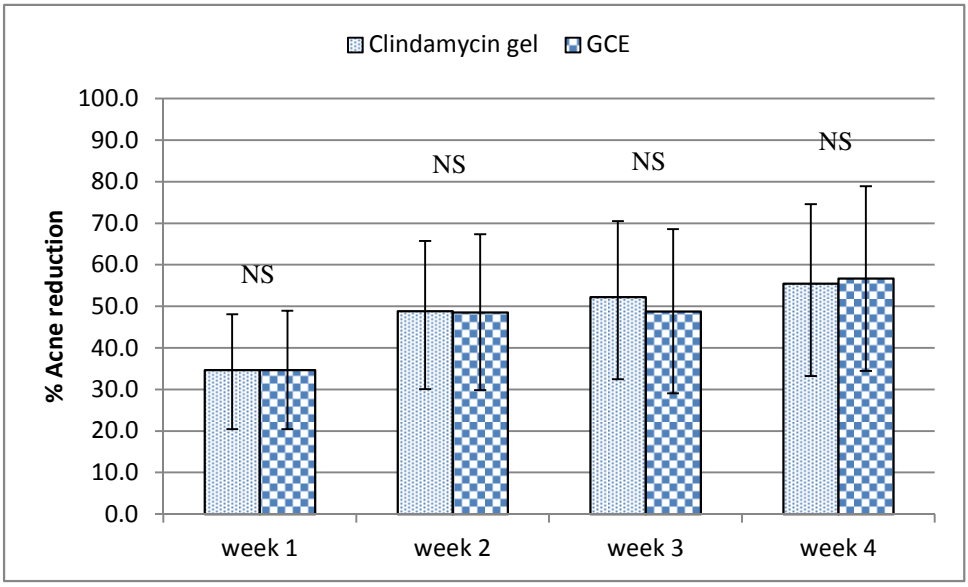


Figure 25 Percentage acne reduction, NS: not different

Adverse effects reported by the patients and accessed by the physicians were recorded (Table 9). Every week of visit the patient reported or the physician assessed the adverse effects. One patient who had mild scaling and mild stinging almost the time but the patient insisted to continue. At the end of study the patient's acne reduced and GAAS score improved. Moderate stinging was reported by a patient who has some nodulocysts at the baseline and GAAS score 4. pH of GCE was determined to be 3.24 while that of the commercial clindamycin gel was around 5.5. Therefore the more acidic pH of GCE could bring the skin uncomfortable.

The acne lesion of each patient was observed at the beginning and every week. Figures 26-29 showed the acne lesion of some male and female patients from both group. The acne lesion was depleted and left over the post-inflammatory hyperpigmentation (PIH).

Table 9 Adverse effects during 1 month study

Adverse effects	Number of case reported (every week)	
	Clindamycin gel	**GCE**
mild scaling	-	4
mild dryness	1	1
mild pruritus	3	2
mild stinging	2	4
moderate stinging	-	1

Further studies should be done to characterize and purify the peak no.6 which is likely related to the antimicrobial activity of ESDE. To increase the stability of the formulation, the cold storage with another gelling agent apart from Poloxamer should be applied. To diminish the adverse effect of GCE, the new application method such as apply for a period of time and then rinse off should be considered.

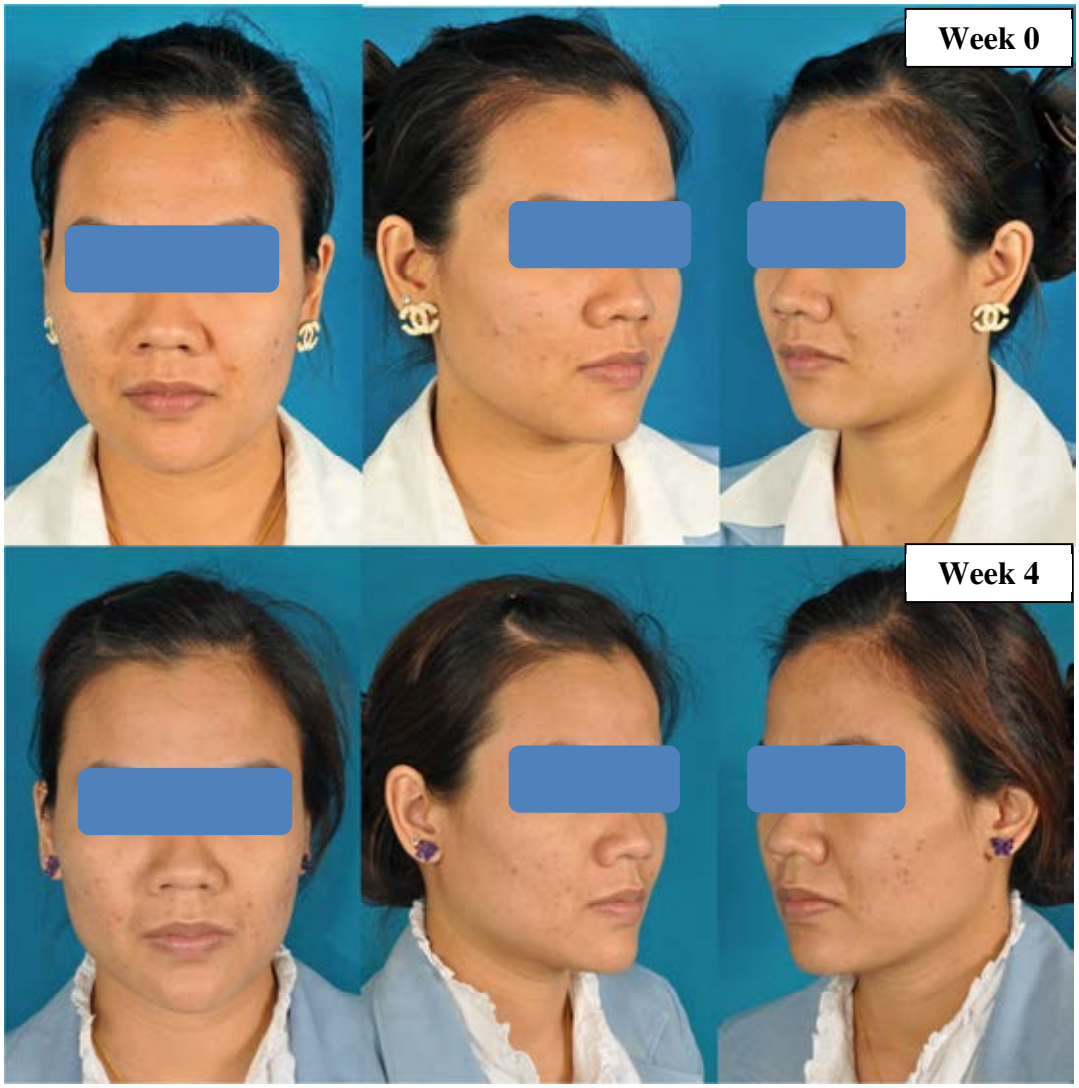


Figure 26 Picture of patients before and after treatment; A 28 year, female patient, received 1% clindamycin gel; week 0: papule 15, pustule 2, GAAS 3, week 4: papule 4, pustule 0, GAAS 2

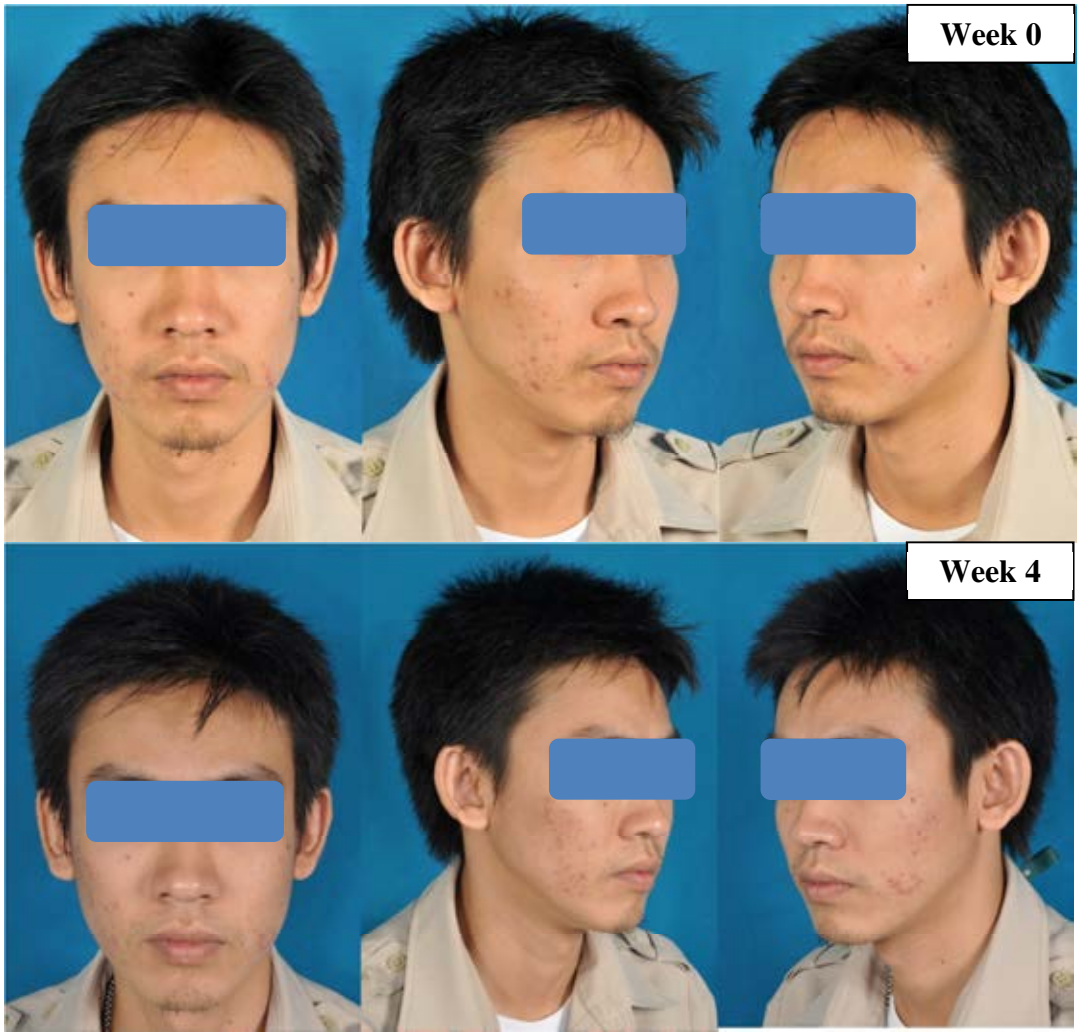


Figure 27 Picture of patients before and after treatment**;** A 25 year, male patient, received 1% clindamycin gel; week 0: papule 10, pustule 2, GAAS 3, week 4: papule 5, pustule 1, GAAS 3

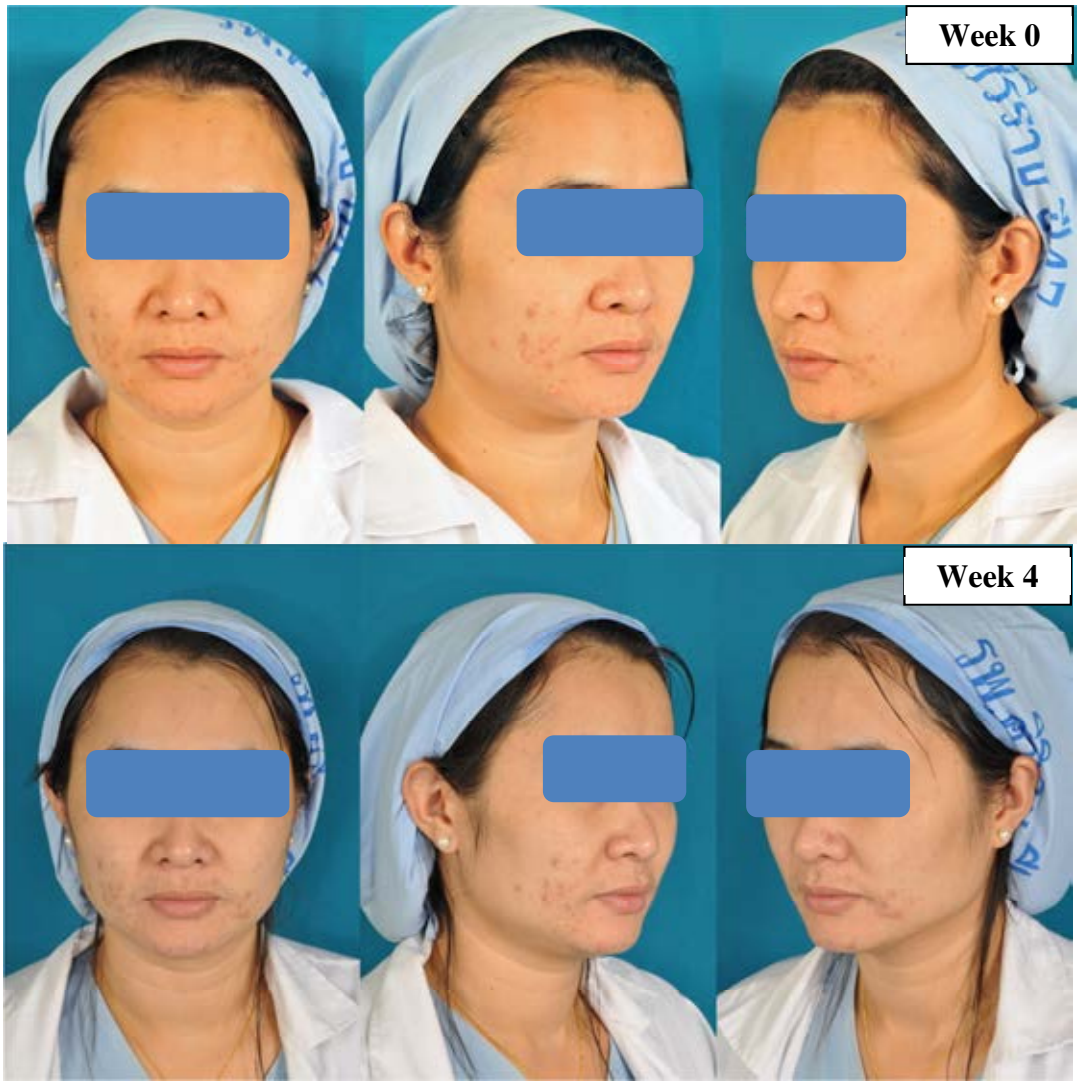


Figure 28 Picture of patients before and after treatment**;** A 37 year, female patient, received GCE; week 0: papule 14, pustule 0, GAAS 3, week 4: papule 4, pustule 0, GAAS 2

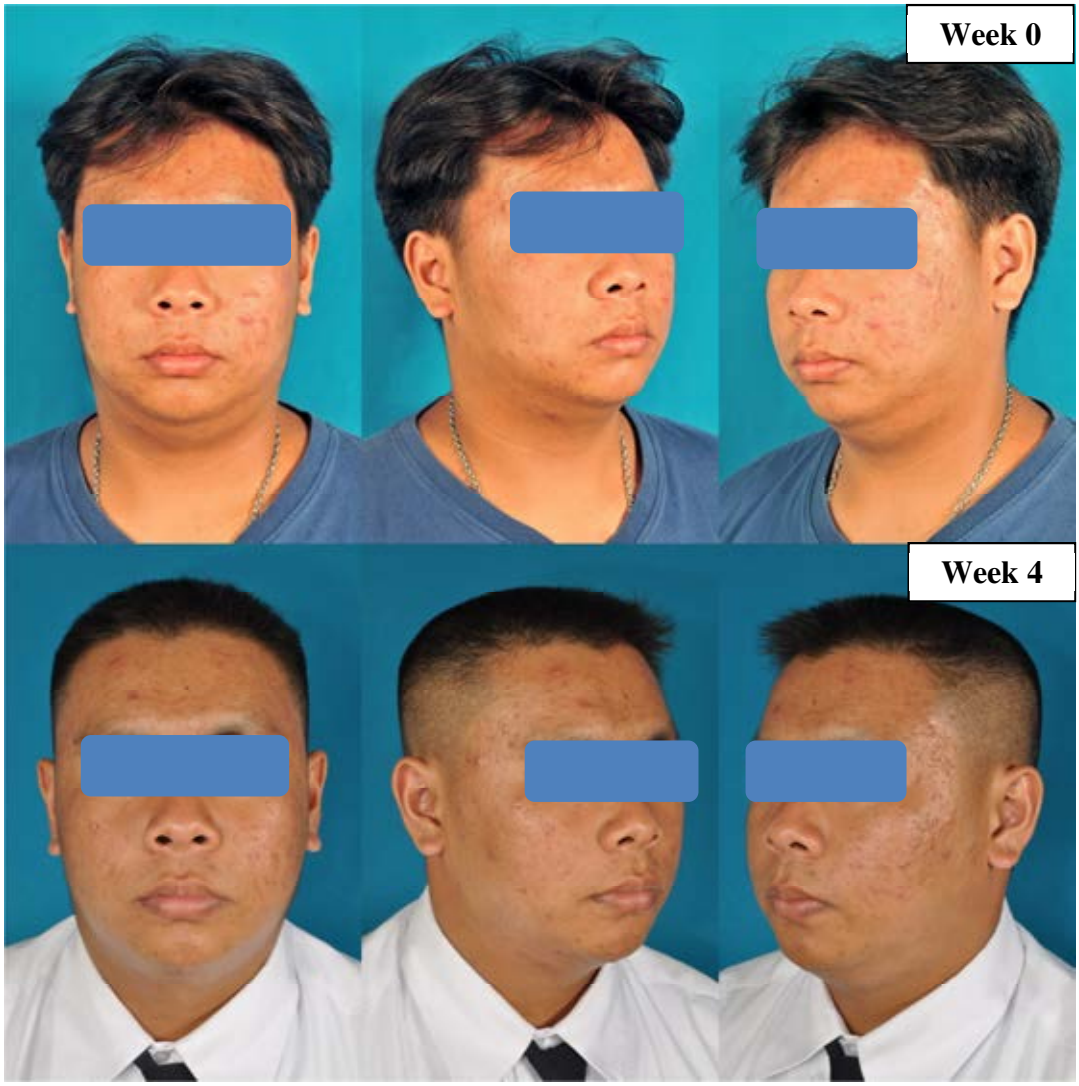


Figure 29 Picture of patients before and after treatment**;** A 18 year, male patient, received GCE; week 0: papule 13, pustule 0, GAAS 3, week 4: papule 8, pustule 2, GAAS 3

CHAPTER V
CONCLUSION

In this research two different sources of emblica fruit extract powder (EFEP), SNP and ESDE, were extracted with acetone, methanol and ethyl acetate separately. The marc from ethyl acetate was continually extracted with acetone and methanol, respectively. Five fractions from each EFEP were obtained i.e. AC, MN, EA, ACS and MNS fractions. They were viscous with the color variation from yellow, light brown to dark brown. The fraction yields from ESDE were higher yield than the corresponding yields of SNP's. The percentage yield of the fractions increased when the polarity of extraction solvent increased. It could be implied that the chemical constituents of EFEP were soluble in water since methanol gave the highest yield.

SNP, ESDE and their fractions were characterized their chemical contents and antimicrobial activities. By HPLC technique, the major compound present in SNP and its fractions was gallic acid. The chromatogram of ESDE and its fractions showed at least six major compounds with one was assigned to be gallic acid. EA fractions showed to contain highest amount of gallic acid and total phenolic compounds. Although fractions gave higher phenolic compound than that of ESDE, it was not chosen to be further formulation study since the viscous resin-like property and the intense color. In this study the ESDE was chosen to be formulated in the gel preparation.

The ESDE showed antimicrobial activity against 30 isolates of *S. aureus*, one ATCC and 29 clinical isolates. ESDE showed wider inhibition zone than TC when the bacteria were considered to be resistant to TC. On the contrary, ESDE showed narrower inhibition zone than that of TC to isolates which were sensitive to TC. The minimum inhibitory concentration (MIC) of ESDE against 30 isolates of *S. aureus* was 10 mg/mL.

Due to the instability of phenolic compound in emblica extract (Chinsuwan and Siritientong, 2008), citrate buffer pH 3.0 (CS) was chosen in this study because it could control constant pH and showed the slowest increase in gallic acid content over 1 month storage at 30 ^{o}C. The peak area and time profile of ESDE showed six peaks. Interestingly, peak no.6 decreased dramatically within a week coincidentally with the decreasing of antimicrobial activity.

Gel containing 10% emblica extract (GCE) was formulated in the presence of 0.15% sodium benzoate and 0.15% sodium metabisulfite in citrate buffer pH 3.0. GCE was yellow translucent gel while the gel blank (BLA) was clear. The color of gel changed to brown color within a week and eventually to dark brown color within a month. The pH was observed to be 3.24 $\pm$ 0.06 over one month storage at 30 ^{o}C. The viscosity of GCE changed from 1238 $\pm$ 75.60 at day 7 to 1040 $\pm$ 21.79 cP at day 27. Gallic acid content increased from 0.06 at day 0 to 0.09 % w/w at day 28. The antimicrobial activity of GCE against 30 strains of *S. aureus* at day 0 and day 28 was declined.

Anti-acne efficacy of GCE was compared with that of 1% clindamycin on the 25 patients. At the end of each week, means acne reduction between two groups were not different ($p>0.05$). The individual acne reduction from week 0 to week 4 was significantly different in both groups ($p<0.05$). The adverse effects of GCE were mild scaling, mild dryness, mild pruritus and mild to moderate stinging.

ESDE has the antimicrobial activity against 30 strains of *S. aureus* including sensitive and resistant isolates to TC. At least five phenolic compounds and gallic acid were detected. The compound detected at retention time 11.9 minute was likely to relate to the decrement of antimicrobial activity. Further study should be done to purify and characterize this compound. The cold storage of GCE may be chosen to preserve the gel color. However, another gelling agent apart from Poloxamer should be applied. To eliminate the adverse effects, the new application method such as apply and rinse off should be considered.

Appendix A

Preparation of emblica extract and its fractions

Table A1 Preparation of emblica fruit extract powder and extraction with different solvent

Fraction	SNP			EFF		
	Input (kg)	**Output (kg)**	**%Yield**	**Input (kg)**	**Output (kg)**	**%Yield**
SD	N/A	N/A	N/A	115	2.200	1.91
AC	1	0.00078	0.078	1	0.423	42.28
MN	0.5	0.073	14.66	0.5	0.378	75.63
EA	3	0.00355	0.12	0.3	0.049	16.48
ACS	N/A	0.00763	0.25[a]	N/A	0.011	3.79[a]
MNS	N/A	0.306	10.20[a]	N/A	0.036	11.99[a]

[a] calculated from the input of EA

Table A2 The physical and chemical properties of ESDE Lot 2

Test	Specification	Test Result	Conclusion
physical appearance	slightly yellow with specific odor	Slightly yellow with special odor	Conformed
pH (1% in water)	2.5-3.5	2.96	Conformed
% water content	less than 6.0	3.999 %	Conformed
% loss on drying	less than 7.0	5.65 $\pm$ 0.15 %	Conformed
As (ppm)	less than 0.07	Nd (LOD=0.02 ppm)	Conformed
Pb (ppm)	less than 3.0	Nd (LOD=0.13 ppm)	Conformed
organic volatile impurities (%w/w)	not found	N/A	N/A
% sulfate	less than 2.0	0.38 %	Conformed
identification (HPLC)	conform	N/A	N/A
assay			
total tannin (%GAE)	more than 20.0	15.77 $\pm$ 0.33	Fail
gallic acid (%w/w)	less than 1.0	0.31%	Conformed

Nd=Not detected, N/A=Not available

Appendix B

Characterization of emblica crude extract and fractions

Table 1B1 Standard gallic acid

Concentration (µg/ml)	Peak area	Retention time (minute)
10	635080	4.71
25	1583056	4.72
50	3161746	4.72
75	4638962	4.73
100	6172584	4.74

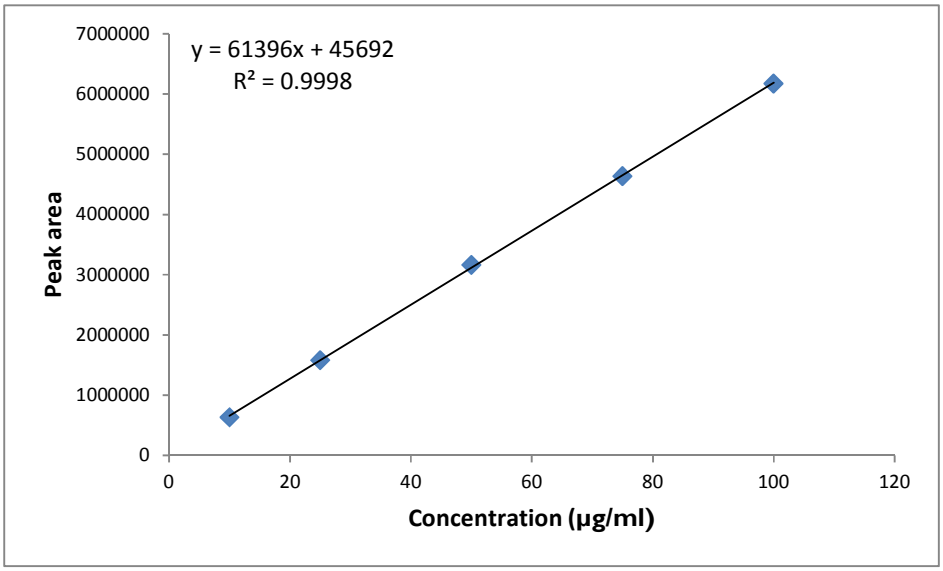


Figure 1B1 Standard curve of gallic acid

Table 1B2 Peak area and gallic acid content in SNP and its fractions

Fractions	Peak area	GAE* (µg/mL)	Concentration (µg/mL)	% Gallic acid (w/w)
SNP	2777462	44.5	5112	0.87
AC	4455898	71.8	486	14.77
MN	121959	nd	40.8	nd
EA	1201299	18.8	111.8	30.03
ACS	109033643	nd	676	nd
MNS	240684	nd	67.2	nd

* calculated from the standard curve of gallic acid concentration 10-100 µg/ml

Nd = can not calculate from the exist standard curve

HPLC method validation

Linearity

Standard gallic acid solutions were prepared at the final concentration of 4, 8, 16, 24 and 32 µg/mL. Then each of concentration was analyzed by HPLC. The peak area obtained and the concentration were plotted to construct the calibration curve. The regression equation and the correlation coefficient (R^2) were calculated.

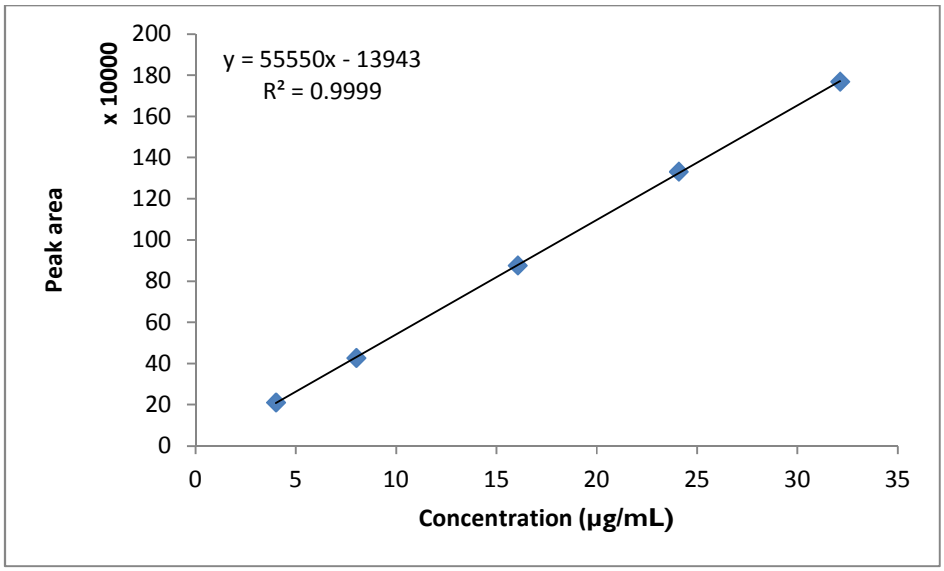


Figure 2B1 The standard curve of gallic acid concentration 4-32 µg/mL

Recovery

Certain amount of gallic acid was added to three concentrations of gallic acid solution. The peak area obtained were calculated from the regression equation and reported as concentration. The concentration obtained from the calculation and the exact adding amount was compared.

Table 2B1 The percentage recovery of gallic acid

Conc (µg/ml)	% Recovery			Mean ± SD
	1	2	3	
12.0144	99.77	99.29	98.73	99.27 ± 0.5182
20.0240	99.08	99.67	98.89	99.21 ± 0.4101
28.0336	99.72	99.50	99.46	99.56 ± 0.1394

Intra-day precision

Three concentrations of gallic acid were analyzed by HPLC within a day. Each concentration was analyzed five times. The concentration that obtained from calculation was collected. Mean and standard deviation of the concentration are listed. The relative standard deviation of five run was calculated and reported.

Table 2B2 The intra-day precision of gallic acid solution

Conc (µg/ml)	1	2	3	4	5	Mean ± SD	% RSD
12.0240	12.0975	12.0370	12.0293	12.0574	12.0793	12.0601±0.0285	0.24
20.0400	20.1224	20.1518	20.1988	20.1986	20.1789	20.1701±0.0329	0.16
28.0560	27.9776	28.0951	28.1226	28.1144	28.0018	28.0623±0.0675	0.24

Inter-day precision

Three concentrations of gallic acid were analyzed by HPLC in the same manner as the intra-day precision. But for inter-day precision, the analysis was performed in three different days.

Table 2B3 The inter-day precision of gallic acid solution

No.	Conc (µg/ml)	1	2	3	4	5	Mean ± SD	%RSD
1	12.0240	12.0975	12.0370	12.0293	12.0574	12.0793	12.0601±0.0285	0.24
	20.0400	20.1224	20.1518	20.1988	20.1986	20.1789	20.1701±0.0329	0.16
	28.0560	27.9776	28.0951	28.1226	28.1144	28.0018	28.0623±0.0675	0.24
2	12.0000	12.0547	11.9177	12.0536	12.0153	12.0383	12.0160±0.0571	0.48
	20.0000	19.8933	19.8239	19.9657	19.9332	19.8353	19.8903±0.0612	0.31
	28.0000	27.6414	27.6293	27.5606	27.7147	27.6966	27.6490±0.0609	0.22
3	12.0144	11.9867	11.9859	11.9545	11.9391	11.9038	11.9540±0.0348	0.29
	20.0240	19.8398	19.7814	19.8429	19.8764	19.8130	19.8307±0.0356	0.18
	28.0336	27.9557	27.9835	27.9972	27.9908	27.9519	27.9758±0.0207	0.07

Table 2B4 Gallic acid content of ESDE and its fraction

Fractions	Concentration (µg/ml)	Peak area	GAE (µg/ml)	% Gallic acid (w/w)
ESDE	2044.4	334082	6.265	0.31
AC	202	933703	17.06	8.44
MN	2020	1034354	18.87	0.93
EA	80.96	618464	11.38	14.06
ACS	202	753839	13.82	6.84
MNS	2040	738647	13.55	0.66

* calculated from the standard curve of gallic acid concentration 4-32 µg/ml

Table 3B1 The absorbance at 747 nm of the reaction mixture containing gallic acid

Concentration (µg/ml)	Absorbance at 747 nm
8	0.048
20	0.186
40	0.414
60	0.626
80	0.840
100	1.048

Table 3B2 Percent phenolic compounds present in ESDP and its fractions

Fraction	Concentration (µg/ml)	Absorbance 747 nm	GAE (µg/ml)	% total phenolics	Mean ± SD
ESDE	98.2	0.135	15.18	15.46	15.77 ± 0.3281
		0.142	15.83	16.12	
		0.138	15.46	15.74	
MN	118.8	0.221	23.07	19.42	19.58 ± 0.1544
		0.223	23.26	19.58	
		0.225	23.44	19.73	
AC	87.6	0.198	20.96	23.93	23.97 ± 0.0605
		0.199	21.06	24.04	
		0.198	20.96	23.93	
EA	99.4	0.295	29.86	30.04	29.92 ± 0.2132
		0.295	29.86	30.04	
		0.291	29.50	29.67	
ACS	106	0.176	18.94	17.87	17.96 ± 0.0866
		0.177	19.04	17.96	
		0.178	19.13	18.05	
MNS	105.6	0.167	18.12	17.16	17.39 + 0.2654
		0.173	18.67	17.68	
		0.169	18.30	17.33	

Table 4B1 Antimicrobial activity of ESDE against 30 strains of *S. aureus*

No.	*S. aureus* isolates no.	Inhibition zone (mm) Experiment I (n=3)		Inhibition zone (mm) Experiment II (n=2)			
		ESDE 10 mg/disc	Tetracycline 30 µg/disc	ESDE 2 mg/disc	Clindamycin		
					2 µg/disc	0.2 µg/disc	0.02 µg/disc
1	ATCC 25922	11.90 ± 0.05	25.63 ± 0.49	9.26	22.16	13.41	8.86
				9.57	21.24	14.81	6.75
2	573	12.36 ± 0.14	29.83 ± 0.18	8.29	15.69	10.60	8.08
				8.23	15.64	11.34	7.29
3	563	13.42 ± 0.61	28.72 ± 0.50	9.35	19.70	12.00	6.43
				9.83	20.56	12.75	6.80
4	572	10.97 ± 0.30	28.64 ± 0.04	8.63	13.14	11.76	7.93
				8.97	14.70	11.41	8.63
5	565	12.77 ± 0.19	27.88 ± 0.14	9.66	16.14	15.23	8.11
				9.92	21.14	14.55	7.90
6	578	12.17 ± 0.44	27.72 ± 0.22	7.90	14.91	11.71	8.89
				9.00	14.30	12.89	8.91
7	587	11.21 ± 0.44	27.64 ± 0.01	9.27	14.86	11.48	7.87
				8.90	17.40	11.24	7.24
8	569	11.59 ± 0.28	27.64 ± 0.08	9.85	0	0	0
				8.75	0	0	0
9	570	11.87 ± 0.13	27.28 ± 0.28	9.49	20.64	13.06	7.88
				9.25	20.42	12.72	8.00
10	577	12.73 ± 0.03	26.71 ± 0.38	10.05	19.06	13.44	8.36
				11.04	21.25	13.99	8.23
11	590	11.07 ± 0.49	26.33 ± 0.38	9.04	21.29	14.52	6.72
				10.04	23.18	13.98	7.63

Table 4B1 (cont) Antimicrobial activity of ESDE against 30 strains of *S. aureus*

No.	*S. aureus* isolates no.	Inhibition zone (mm) Experiment I (n=3)		Inhibition zone (mm) Experiment II (n=2)			
		ESDE 10 mg/disc	Tetracycline 30 µg/disc	ESDE 2 mg/disc	Clindamycin		
					2 µg/disc	0.2 µg/disc	0.02 µg/disc
12	576	11.58 ± 0.23	25.77 ± 0.05	8.49	13.89	10.55	6.30
				8.35	14.74	10.96	7.47
13	592	12.94 ± 0.61	25.53 ± 0.88	10.08	21.59	14.06	9.05
				10.07	20.90	13.01	7.00
14	584	13.47 ± 0.01	25.53 ± 0.16	9.20	21.48	14.64	8.64
				8.87	21.72	15.33	9.74
15	562	11.05 ± 0.55	25.43 ± 1.03	8.04	14.20	10.95	7.28
				8.52	12.33	9.75	6.24
16	564	12.59 ± 0.49	24.77 ± 0.18	9.59	20.59	12.50	6.97
				9.31	21.15	14.26	7.74
17	582	13.78 ± 0.22	24.66 ± 0.69	10.42	21.29	15.79	7.27
				10.53	21.49	15.73	7.22
18	589	12.18 ± 0.08	23.82 ± 0.06	9.97	22.99	15.55	8.25
				10.47	21.81	15.78	7.85
19	575	13.62 ± 0.28	23.27 ± 0.06	9.20	20.85	15.76	9.81
				9.83	14.38	15.24	9.35
20	568	10.50 ± 0.40	10.56 ± 0.16	8.54	20.33	13.94	6.74
				9.03	19.56	12.43	8.11
21	591	11.95 ± 0.09	10.02 ± 0.04	8.84	20.15	12.34	8.03
				9.33	18.89	12.30	8.13
22	580	10.55 ± 0.05	8.96 ± 0.11	8.47	19.87	14.30	7.78
				9.30	18.07	14.07	7.06

Table 4B1 (cont) Antimicrobial activity of ESDE against 30 strains of *S. aureus*

No.	*S. aureus* isolates no.	Inhibition zone (mm) Experiment I (n=3)		Inhibition zone (mm) Experiment II (n=2)			
		ESDE 10 mg/disc	Tetracycline 30 µg/disc	ESDE 2 mg/disc	Clindamycin		
					2 µg/disc	0.2 µg/disc	0.02 µg/disc
23	579	10.99 ± 0.35	8.90 ± 0.10	8.61	14.03	10.62	7.87
				8.36	15.23	10.20	7.24
24	567	11.72 ± 0.13	8.72 ± 0.35	8.90	13.85	9.87	6.29
				7.86	13.36	9.88	7.25
25	588	12.90 ± 0.06	8.38 ± 0.08	9.22	20.28	13.91	7.92
				9.43	21.00	14.82	8.95
26	571	12.82 ± 0.88	8.30 ± 0.00	9.64	21.25	13.65	8.36
				9.54	21.57	14.17	8.72
27	586	12.61 ± 0.12	8.29 ± 0.40	10.16	19.93	13.54	7.84
				9.63	21.04	14.53	7.90
28	581	12.58 ± 0.01	7.73 ± 0.18	8.84	21.13	14.15	8.06
				8.83	20.79	13.70	8.05
29	585	13.60 ± 0.40	7.70 ± 0.08	9.23	24.72	14.39	8.00
				9.46	23.70	14.60	6.77
30	593	9.20 ± 0.22	7.25 ± 0.05	6.38	22.41	12.65	6.41
				7.69	20.80	14.83	7.28

Table 4B2 Minimum Inhibitory Concentration (MIC) of ESDE against 30 strains of *S. aureus*

S.aureus*	**MIC (mg/ml)**	***S.aureus****	**MIC (mg/ml)**	***S.aureus****	**MIC (mg/ml)**
ATCC 25923	5	591	5	589	5
578	5	580	5	582	5
572	5	570	5	586	5
587	5	563	5	581	5
573	5	568	5	564	5
567	5	590	5	585	5
579	5	592	5	584	5
576	5	577	5	593	10
562	5	565	5	571	5
569	5	575	5	588	5

Appendix C

Development of gel containing emblica extract

Table 1C1 pH value of the emblica extract in Citrate buffer (CS), Phosphate buffer (PS), water that adjusted pH equal to 3 (WSR) and in water (WS)

Day	CS	PS	WSR	WS
0	3.29 ± 0.02	3.65 ± 0.10	3.26 ± 0.01	3.72 ± 0.06
6	3.15 ± 0.06	3.38 ± 0.09	3.25 ± 0.05	3.47 ± 0.08
14	3.15 ± 0.10	3.54 ± 0.11	3.49 ± 0.03	3.60 ± 0.03
22	3.27 ± 0.11	3.61 ± 0.08	3.55 ± 0.06	3.69 ± 0.06
29	3.30 ± 0.08	3.69 ± 0.09	3.65 ± 0.09	3.80 ± 0.05
mean ± SD	3.23 ± 0.08	3.58 ± 0.12	3.44 ± 0.18	3.65 ± 0.13

Table 1C2 The gallic acid content of the emblica extract in Citrate buffer (CS), Phosphate buffer (PS), water adjusted pH to 3.0 with 1N HCl (WSR) and in water (WS)

Day	% Gallic acid (w/w)			
	CS	**PS**	**WSR**	**WS**
0	1.20 ± 0.09	1.17 ± 0.09	1.21 ± 0.08	1.15 ± 0.14
1	1.29 ± 0.17	1.38 ± 0.08	nd	1.48 ± 0.08
2	nd	nd	1.27 ± 0.04	nd
4	1.26 ± 0.12	1.47 ± 0.10	1.55 ± 0.24	1.48 ± 0.04
6	1.32 ± 0.04	1.38 ± 0.06	nd	1.49 ± 0.03
8	nd	nd	1.74 ± 0.06	nd
18	1.73 ± 0.16	2.05 ± 0.13	nd	2.14 ± 0.14
22	nd	nd	2.05 ± 0.04	nd
29	2.03 ± 0.04	2.26 ± 0.07	2.04 ± 0.04	2.40 ± 0.16
Mean	1.47	1.62	1.64	1.69
SD	0.33	0.43	0.37	0.48

Table 1C3 The peak area and time profile of ESDE in Citrate buffer pH 3.0 (a), Phosphate buffer pH 3.0 (b), Water adjust pH to 3.0 with 1N HCl (c) and Water (d)

a)

Day	0	1	4	6	18	29
Peak No.1	3262876	4085696	5406632	5369682	5969264	5891346
Peak No.2	1508740	1388274	1212501	1065859	886329	901319
Peak No.3	2554803	2458484	2639840	2542909	2673249	2547277
Gallic acid	1183894	1161192	1348251	1372376	1794052	1934648
Peak No.5	466317	361475	222857	220790	180016	258289
Peak No.6	1108356	656691	214165	186616	139151	158209

b)

Day	0	1	4	6	18	29
Peak No.1	3360908	4203423	5204838	5477147	5888078	6343150
Peak No.2	1493331	1352860	991512	904002	714156	745634
Peak No.3	2451289	2484370	2420349	2433508	2466628	2513419
Gallic acid	1046831	1215396	1407609	1509737	1856084	2156327
Peak No.5	449872	361948	128651	121202	136942	166008
Peak No.6	1011239	621113	148002	145103	145208	109628

c)

Day	0	2	4	7	22	29
Peak No.1	3877804	4931238	5428974	6301383	6421753	5688490
Peak No.2	1809339	1433972	1272456	1096030	946567	737397
Peak No.3	2828915	2671243	2713324	2824022	2704371	2409113
Gallic acid	1253549	1302419	1421891	1768545	1968418	1869986
Peak No.5	518199	389423	255313	145313	193598	162655
Peak No.6	1260098	648994	272752	134469	144906	110017

Table 1C3 (cont) The peak area and time profile of ESDE in Citrate buffer pH 3.0 (a), Phosphate buffer pH 3.0 (b), Water adjust pH to 3.0 with 1N HCl (c) and Water (d)

d)

Day	0	1	4	6	18	29
Peak No.1	3854904	4338827	5614027	6007327	6754259	6735952
Peak No.2	1674190	1517218	1372137	1242644	882034	906298
Peak No.3	2739162	2789738	2813236	2776308	2822847	2787887
Gallic acid	1206838	1446490	1537117	1522762	2245694	2484499
Peak No.5	495741	494489	334725	264526	160387	197778
Peak No.6	1155913	770630	281230	262689	149299	139326

Table 2C3 pH value of emblica gel (GCE) and gel without emblica extract (BLA) over 1 month storage at 30 ^{o}C

Day	GCE1	GCE2	GCE3	BLA1	BLA2	BLA3
0	3.28	nd	nd	3.55	nd	nd
5	3.28	nd	nd	3.63	nd	nd
7	3.2	3.19	3.2	3.58	3.52	3.54
14	3.23	3.21	3.22	3.55	3.58	3.56
20	3.2	3.19	3.18	3.5	3.49	3.48
27	3.34	3.18	3.21	3.49	3.47	3.49

nd = not determine

Table 2C4 Viscosity of GCE and blank gel over 1 month storage at 30 ^{o}C

Day	GCE1	GCE2	GCE3
0	1899.00 ± 23.61	nd	nd
7	1268.00 ± 75.60	985.63 ± 55.02	1042.33 ± 26.10
13	1152.00 ± 86.28	996.67 ± 41.88	862.70 ± 27.94
21	1012.93 ± 53.30	952.17 ± 50.43	739.17 ± 24.11
27	1040.00 ± 21.79	871.47 ± 34.43	743.47 ± 21.59
Day	**BLA1**	**BLA2**	**BLA3**
0	2118.60 ± 52.10	nd	nd
7	2091.00 ± 10.39	740.00 ± 14.55	1658.33 ± 29.54
13	2122.33 ± 11.02	153.50 ± 6.62	143.33 ± 4.97
21	105.90 ± 6.68	109.77 ± 2.06	117.40 ± 3.44
27	107.10 ± 5.77	132.73 ± 2.02	118.90 ± 1.87

nd = not determine

Table 2C5 Percentage of gallic acid in GCE over 1 month storage at 30 ^{o}C,

DAY	% Gallic acid (w/w)							
	GCE 1		GCE 2		GCE 3		mean	SD
	1	2	1	2	1	2		
0	0.06	0.06	0.07	0.07	0.06	0.07	0.07	0.01
5	0.06	0.06	0.07	0.07	0.07	0.07	0.07	0.01
7	0.08	0.08	0.08	0.08	0.08	0.07	0.08	0.00
14	0.08	0.08	0.08	0.08	0.07	0.07	0.08	0.01
21	0.08	0.08	0.09	0.09	0.08	0.08	0.08	0.01
28	0.09	0.09	0.09	0.09	0.08	0.08	0.09	0.01

Table 2C6 Peak area and time profile of GCE over 1 month storage at 30 ^{o}C

Peak	Day 0			Day 5			Day 7			Day 14			Day 20			Day 27		
no.	PA	RT	relative RT	PA	RT	relative RT	PA	RT	relative RT	PA	RT	relative RT	PA	RT	relative RT	PA	RT	relative RT
1	3968732	4.1	1.0	4406894	3.6	1.0	3891804	3.4	1.0	4567020	3.4	1.0	4226903	3.3	1.0	4655458	3.4	1.0
2	1299216	6.5	1.6	1154213	5.7	1.6	898516	5.3	1.6	1019711	5.5	1.6	899893	5.1	1.5	892207	5.4	1.6
3	2273001	8.1	2.0	2251105	7.2	2.0	1838076	6.7	2.0	2224110	6.9	2.0	2017565	6.8	2.0	2114967	6.8	2.0
4	451740	10.2	2.5	443351	9.1	2.5	402673	8.4	2.5	513901	8.6	2.5	484827	8.5	2.6	573752	8.5	2.5
5	218981	12.1	3.0	164095	10.7	2.9	141858	10.0	3.0	201933	10.3	3.0	215155	9.8	3.0	250336	10.1	3.0
6	775058	14.3	3.5	637056	12.6	3.5	350114	11.7	3.5	329129	12.0	3.5	291201	11.5	3.5	270797	11.9	3.5

Table 2C7 Antimicrobial activity of 1% clindamycin gel (CLN), GCE and gel blank against 30 strains of *S. aureus* at day 0 and day 28

S. aureus	CLN		GCE		Gel blank	
	Day 0	Day 28	Day 0	Day 28	Day 0	Day28
ATCC 25923	23.77 ± 1.48	23.93 ± 0.99	14.46 ± 1.01	12.04 ± 0.39	13.03	10.74
562	26.88 ± 1.85	20.16 ± 1.17	12.62 ± 1.51	9.96 ± 0.91	12.33	8.37
563	23.50 ± 3.39	21.71 ± 1.96	15.12 ± 3.08	11.56 ± 0.47	11.96	9.16
564	24.09 ± 3.59	22.86 ± 1.28	16.49 ± 2.04	13.51 ± 2.12	12.51	8.22
565	7.37 ± 12.77	23.15 ± 0.43	11.16 ± 4.07	12.86 ± 2.76	10.58	8.67
567	25.17 ± 2.23	21.06 ± 2.60	14.58 ± 1.56	9.63 ± 1.57	11.68	8.35
568	25.43 ± 1.98	23.75 ± 2.61	17.19 ± 1.68	12.42 ± 0.52	9.99	7.94
569	0.00 ± 0.00	0.00 ± 0.00	15.10 ± 2.91	10.33 ± 1.22	13.99	8.82
570	22.33 ± 1.67	24.28 ± 1.60	13.59 ± 0.46	12.24 ± 0.67	13.80	9.48
571	23.07 ± 1.84	23.25 ± 0.81	17.10 ± 0.16	12.84 ± 0.38	10.82	10.96
572	22.83 ± 1.39	22.38 ± 1.12	11.40 ± 1.48	10.51 ± 1.78	9.69	9.54
573	22.29 ± 0.65	22.00 ± 1.44	14.98 ± 2.58	11.51 ± 0.97	7.95	9.65
575	25.91 ± 1.69	22.96 ± 0.80	13.29 ± 0.96	9.79 ± 0.61	9.92	9.60
576	27.18 ± 2.45	22.29 ± 1.06	16.12 ± 1.71	9.22 ± 0.81	17.58	9.04
577	23.56 ± 1.60	22.83 ± 0.99	14.78 ± 2.34	11.03 ± 0.73	11.62	9.11
578	21.40 ± 4.68	20.98 ± 1.56	13.23 ± 0.91	11.20 ± 0.76	8.34	9.27
579	25.13 ± 3.04	22.30 ± 0.63	13.00 ± 2.18	11.03 ± 1.12	10.41	10.82
580	26.03 ± 0.67	24.10 ± 2.28	13.99 ± 0.30	11.83 ± 0.66	10.95	7.43
581	24.38 ± 1.82	23.48 ± 1.09	14.63 ± 2.87	11.42 ± 2.86	9.98	8.66
582	23.99 ± 1.34	21.92 ± 0.49	13.94 ± 2.87	10.99 ± 0.50	10.09	8.95
584	22.82 ± 0.72	22.15 ± 1.43	15.46 ± 0.44	10.69 ± 0.54	9.15	10.39
585	24.60 ± 1.47	22.48 ± 0.14	14.66 ± 2.12	11.36 ± 0.77	10.76	8.11
586	24.92 ± 0.66	23.37 ± 0.58	14.07 ± 0.96	12.26 ± 0.58	9.67	10.29
587	22.63 ± 1.18	22.92 ± 1.49	12.22 ± 3.41	11.51 ± 2.35	9.20	9.53
588	25.67 ± 0.65	22.15 ± 1.27	15.05 ± 1.67	11.09 ± 0.56	10.77	7.80
589	23.31 ± 1.65	23.97 ± 0.79	15.46 ± 3.11	11.37 ± 1.51	8.16	8.61
590	25.73 ± 0.58	20.57 ± 1.84	15.20 ± 2.68	11.84 ± 1.85	7.80	10.23
591	25.44 ± 1.25	20.45 ± 1.18	13.45 ± 0.92	10.10 ± 1.12	13.20	10.83
592	24.42 ± 0.61	22.86 ± 0.61	13.39 ± 1.86	11.71 ± 1.95	na	8.22
593	22.33 ± 5.52	25.47 ± 1.15	12.29 ± 0.22	11.18 ± 0.40	na	10.85

na = data not available

Table 2C8 Antimicrobial activity of 1% clindamycin gel (CLN, a), GCE (b) and gel blank (c) against 5 strains of *S. aureus* at day 0, 7, 14, 20 and 28

a)

S.aureus	**day 0**	**day 7**	**day 14**	**day 20**	**day 28**
ATCC 25923	23.77 ± 1.48	24.29 ± 1.35	23.10 ± 2.13	23.24 ± 1.39	23.93 ± 0.99
564	24.09 ± 3.59	24.51 ± 2.35	23.34 ± 3.00	24.30 ± 0.77	22.86 ± **1.28**
569	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00	0.00 ± 0.00
571	23.07 ± 1.84	24.83 ± 1.36	22.75 ± 1.29	24.35 ± 2.33	23.25 ± 0.81
579	25.13 ± 3.04	26.39 ± 0.56	25.37 ± 1.72	23.53 ± 0.76	22.30 ± **0.63**

b)

S.aureus	**day 0**	**day 7**	**day 14**	**day 20**	**day 28**
ATCC 25923	14.46 ± 1.01	12.64 ± 1.65	10.90 ± 0.12	16.38 ± 2.20	12.04 ± 0.39
564	16.49 ± 2.04	11.31 ± 0.35	12.36 ± 1.38	15.60 ± 1.01	13.51 ± 2.12
569	15.10 ± 2.91	11.34 ± 0.43	11.01 ± 0.69	14.40 ± 1.54	10.33 ± 1.22
571	17.10 ± 0.16	13.07 ± 0.62	13.16 ± 0.20	17.50 ± 1.57	12.84 ± 0.38
579	13.00 ± 2.18	12.09 ± 0.17	12.15 ± 1.23	14.89 ± 0.44	11.03 ± 1.12

c)

S.aureus	**day 0**	**day 7**	**day 14**	**day 20**	**day 28**
ATCC 25923	13.03	12.87	10.23	9.6	10.74
564	12.51	10.92	11.98	10.64	8.22
569	13.99	11.06	9.16	9.85	8.82
571	10.82	10.9	11.34	10.79	10.96
579	10.41	11.92	11.92	12.18	10.82

Appendix D

Efficacy test

Table D1 The number of acne (papules and pustules) after using the given gel over one month period

1% Clindamycin gel					
Week	**WK0**	**WK1**	**WK2**	**WK3**	**WK4**
1	15	10	9	7	10
2	13	8	NA	6	5
3	15	5	3	2	5
4	10	5	3	2	2
5	10	8	5	3	3
6	17	10	8	6	4
7	12	6	6	8	6
8	10	5	4	2	3
9	12	7	8	4	6
10	10	6	6	6	4
11	11	8	8	6	7
12	11	5	4	4	4
13	12	9	7	5	6

GCE					
Week	**WK0**	**WK1**	**WK2**	**WK3**	**WK4**
1	10	8	7	7	4
2	13	11	6	6	10
3	17	13	8	7	11
4	25	19	14	9	13
5	10	7	7	5	4
6	10	6	3	4	3
7	11	7	5	7	1
8	14	9	5	7	4
9	10	5	3	7	5
10	10	4	6	2	5
11	15	NA	8	4	6

NA = the patient did not visit

Table D2 GAAS score of patient at week 0 and week 4

Group A	WK 0	WK 4
1	4	3
2	4	4
3	4	3
4	3	2
5	2	1
6	3	2
7	3	3
8	4	2
9	3	2
10	4	2
11	3	3
12	3	2
13	3	3

Group B	WK 0	WK 4
1	4	4
2	3	3
3	3	3
4	4	4
5	4	4
6	4	4
7	3	2
8	3	2
9	3	2
10	3	2
11	4	2

Table D3 Percentage acne reduction

A: 1% Clindamycin gel					**B: GCE**				
No.	**W0-1**	**W0-2**	**W0-3**	**W0-4**	**No.**	**W0-1**	**W0-2**	**W0-3**	**W0-4**
1	33.33	40.00	53.33	33.33	**1**	20.00	30.00	30.00	60.00
2	38.46	NA	53.85	61.54	**2**	15.38	53.85	53.85	23.08
3	66.67	80.00	86.67	66.67	**3**	23.53	52.94	58.82	35.29
4	50.00	70.00	80.00	80.00	**4**	24.00	44.00	64.00	48.00
5	20.00	50.00	70.00	70.00	**5**	30.00	30.00	50.00	60.00
6	41.18	52.94	64.71	76.47	**6**	40.00	70.00	60.00	70.00
7	50.00	50.00	33.33	50.00	**7**	36.36	54.55	36.36	90.91
8	50.00	60.00	80.00	70.00	**8**	35.71	64.29	50.00	71.43
9	41.67	33.33	66.67	50.00	**9**	50.00	70.00	30.00	50.00
10	40.00	40.00	40.00	60.00	**10**	60.00	40.00	80.00	50.00
11	27.27	27.27	45.45	36.36	**11**	NA	46.67	73.33	60.00
12	54.55	63.64	63.64	63.64	**mean**	33.50	50.57	53.31	56.25
13	25.00	41.67	58.33	50.00	**SD**	13.91	14.11	16.43	18.30
mean	41.39	50.74	61.23	59.08					
SD	13.02	15.53	16.02	14.40					

Data analysis

I. Normality test

Group A: 1% Clindamycin gel

Week 1

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
VAR00001	0.130208	13	0.2	0.97359	13	0.933098
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 2

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.137129	12	0.2	0.974	12	0.947868
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 3

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.110073	13	0.2	0.975832	13	0.952847
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 4

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.140926	13	0.2	0.949311	13	0.587883
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Group B: GCE

Week 1

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.152583	10	0.2	0.952861	10	0.702408
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 2

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.116317	11	0.2	0.935349	11	0.467458
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 3

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.14754	11	0.2	0.950976	11	0.656454
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

Week 4

Tests of Normality						
	Kolmogorov-Smirnov(a)			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	df	Sig.
percent_reduc	0.146011	11	0.2	0.972584	11	0.911356
*	This is a lower bound of the true significance.					
a	Lilliefors Significance Correction					

II. Compare mean acne reduction between group A and B

At the end of week 1

Group Statistics

	gr	N	Mean	Std. Deviation	Std. Error Mean
percent_reduc	1.00	13	41.3941	13.02361	3.61210
	2.00	10	34.0446	14.29793	4.52140

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
percent_reduc	Equal variances assumed	.219	.645	1.286	21	.212	7.34942	5.71390	-4.53329	19.23212
	Equal variances not assumed			1.270	18.502	.220	7.34942	5.78709	-4.78521	19.48404

At the end of week 2

Group Statistics

	gr	N	Mean	Std. Deviation	Std. Error Mean
percent_reduc	1.00	12	50.7375	15.52739	4.48237
	2.00	12	49.3883	14.06809	4.06111

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
percent_reduc	Equal variances assumed	.032	.859	.223	22	.826	1.34917	6.04849	-11.19463	13.89297
	Equal variances not assumed			.223	21.789	.826	1.34917	6.04849	-11.20168	13.90001

At the end of week 3

Group Statistics

	gr	N	Mean	Std. Deviation	Std. Error Mean
percent_reduc	1.00	13	61.2292	16.01994	4.44313
	2.00	12	50.3783	18.65902	5.38639

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
percent_reduc	Equal variances assumed	.219	.644	1.564	23	.132	10.85090	6.93848	-3.50244	25.20423
	Equal variances not assumed			1.554	21.807	.135	10.85090	6.98245	-3.63725	25.33904

At the end of week 4

Group Statistics

	gr	N	Mean	Std. Deviation	Std. Error Mean
percent_reduc	1.00	13	59.0777	14.40616	3.99555
	2.00	11	56.2464	18.29961	5.51754

Independent Samples Test

		Levene's Test for Equality of Variances		t-test for Equality of Means						
									95% Confidence Interval of the Difference	
		F	Sig.	t	df	Sig. (2-tailed)	Mean Difference	Std. Error Difference	Lower	Upper
percent_reduc	Equal variances assumed	.248	.623	.424	22	.676	2.83133	6.67426	-11.01024	16.67290
	Equal variances not assumed			.416	18.906	.682	2.83133	6.81232	-11.43184	17.09450

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