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## Stability of Epigallocatechin Gallate (EGCG) from Green Tea (*Camellia sinensis*) and its Antibacterial Activity against *Staphylococcus epidermidis* ATCC 35984 and *Propionibacterium acnes* ATCC 6919

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### ABSTRACT

One of problem from anti acne cream formula containing Epigallocatechin gallate (EGCG) from green tea leaves is EGCG stability. The EGCG is unstable to heat and easily oxidated. However, EGCG is the main marker substance related to antibacterial activity. This study aimed to determine the EGCG stability and its antibacterial activity. The EGCG stability testing was obtained during extraction process by adding phosphoric acid buffer, pH 4 buffer solution and control. The stability of these groups then tested at 2, 25 and 40°C temperature. Evaluation for absorbance changing by UV-VIS spectrophotometry was obtained during 15 days. After that, EGCG concentration from the three groups were compared using HPLC. Antibacterial activity testing for acne causing bacteria *Staphylococcus epidermidis* ATCC 35984 was conducted from phosphoric acid and pH 4 buffer solution groups. The result showed that extreme cold temperature decreasing treatment against green tea leaves extract after infundation, pH 4 buffer solution adding and storage at temperature of 2°C produced the highest EGCG concentration namely 60.98% w/w. Besides that, antibacterial activity from the ethyl acetate fraction of green tea leaves extract by using this method produced a good inhibition against *P. acnes* and *S. epidermidis*. Moreover, the antibacterial activity against *P. acnes* was higher than *S. epidermis*. The adding of pH 4 buffer solution in extraction process produced the highest EGCG concentration than phosphoric acid adding and no buffer adding. The EGCG was stable at the storage temperature of 2°C and 6% ethyl acetate fraction of green tea leaves extract can be used as an anti acne dosage.

**Key words:** *Camellia sinensis*, EGCG stability, antibacterial, *S. epidermidis* ATCC 35984, *Propionibacterium acnes* ATCC 6919

### INTRODUCTION

Epigallocatechin gallate (EGCG) is easily oxidated. The previous study stated about EGCG on 8 green tea products that circulated in USA, Japan and Korea showed a decrease in EGCG concentration after had been stored in dry temperature 20°C during 6 months because of oxidation (Friedman *et al.*, 2009). The EGCG widely known unstable in alkali pH. The EGCG has better solubility in pH 4-6 and unstable in pH>8. It can be concluded that EGCG stability is affected by pH because acid condition makes EGCG more stable (Lestari and Trisusilawati, 2010).

On the other hand, EGCG is unstable to light. EGCG was degraded up to 85% after exposed by radiation for 1 h. Moreover, EGCG is unstable when heated. Therefore, the infundation process must be conducted in extreme cold and pH adjustment with phosphoric acid until reaching pH 4 Hirun and Roach (2011). This treatment could increase EGCG concentration for 16% higher than methanol solution, while the green tea after infundation process that underwent the temperature decreasing process until 22°C showed that EGCG concentration was 30% lower than methanol solvent. Green tea that underwent 90°C infundation process during 30 min without pH decreasing process showed the same result like the methanol solvent. This study showed the importance of extreme decreasing temperature treatment and decreasing pH to 4 after infundation.

The EGCG stability and concentration were affected by extraction process. In meta analysis study, when cold extreme decreasing and buffer adding had been done in Sugihartini (2013), EGCG concentration result was 6.7%. In Prayong *et al.* (2007) EGCG concentration result was 0.21-9.63%, while in Martono and Martono (2012) EGCG concentration result in brewed green tea was 2.08-3.96%. Besides that, in Hirun and Roach (2011) EGCG concentration with cold extreme decreasing and phosphoric acid adding until pH 4 was 35%.

The EGCG is the most responsible substance related to antibacterial activity. The EGCG could change protein surface polarity and inhibit  $\beta$ -ketoasil (protein transport-asil) reductase reversibility from bacteria, modified protein enzym followed by agregation so it could kill bacteria (Li *et al.*, 2006). The EGCG in green tea not only killed the bacteria but had depigmentation activity so EGCG could rejevunate the blackened skin which caused by acnes (Akhtar *et al.*, 2011; Mahmood *et al.*, 2010).

## **MATERIALS AND METHODS**

**Green tea extract stability test:** Extract stability test was measured by maximum wave length ( $\lambda$ ) in extract that evaluated for 15 days in various temperature. The temperature for testing condition were 5, 25 and 45°C using modified Muehlbach *et al.* (2006) method.

There are 3 groups of experiment:

- First group, green tea extract processed by 90°C infundation for 30 min and given cold extreme treatment and phosphoric acid adjustment until pH 4 (according to Hirun and Roach (2011)). Extract then fraction by ethyl acetate and evaporated until got dry powder
- Second group, green tea extract processed by 90°C infundation for 30 min and given cold extreme treatment and pH 4 buffer solution adjustment (mix of citric acid, HCl and NaOH). Extract then fraction by ethyl acetate and evaporated until got dry powder
- Third group or control. The control was green tea extract processed by 90°C infundation for 30 min and given cold extreme treatment and no buffer adding. Extract then fraction by ethyl acetate and evaporated until got dry powder

All groups have been storage in 2, 25 and 45°C temperature and absorbance value was measured for 15 days.

Ethyl acetate fraction specification in green tea extract: Ethyl acetate fraction specification in green tea extract done by EGCG used Martono and Martono (2012) method which was HPLC reversed phase with isocratic elution system. The mobile phase was a combination of 0.1% phosphoric acid : methanol : acetonitrile : aquabidest in 14:1:3:7 (V/V/V/V). Double distilled water

was used. In addition, after the ingredients were mixed then followed by triethylamine and adjusted to pH 4. The sample was injected with 20  $\mu\text{L}$  inject volume and elected with C 18 stationary phase, flow rate 1.2  $\text{mL min}^{-1}$  and detected with wave length ( $\lambda$ ) 280 nm wavelength with UV spectrophotometer.

**Antibacterial activity:** Antibacterial activity in this study was conducted using diffusion method according to modified Niyomkam *et al.* (2010) research.

*Staphylococcus epidermidis* ATCC 35984 incubated in blood agar plates for 24 h in aerobic condition, bacterial suspension used sterile NaCl 0.9%. Sterile cotton bud was soaked into tube of bacteria then swabbed to Mueller Hinton plates that already incubated for 2 h then made a hole to Mueller Hinton plate with sterile iron. In addition, ethyl acetate green tea extract was added with 1-6% concentration and incubated in 37°C temperature for 24 h.

*Propionibacterium acnes* ATCC 6919 is incubated in blood agar media for 72 h under anaerobic condition and then made the bacterial suspension using sterile 0.9% NaCl. Turbidity cells McFarland synchronized with the standard 0.5 or 0.05 mL BaCl<sub>2</sub> 1% mixed with 9.95 mL of 1% H<sub>2</sub>SO<sub>4</sub>. Sterile cotton stick was inserted into the tube containing the bacteria, then pressed-emphasized in the tube wall from getting too wet. The cotton then swabbed on Mueller Hinton adding sheep blood media which had previously been incubated for approximately 2 h until flat and thin as possible, then created pitting on Mueller Hinton media by using a sterile metal on the media with extract concentration of 1-6%. The plates then were incubated at 37°C for 72 h under anaerobic conditions. Flow study are presented in chart 1.

**Measurement:** Three groups were given treatment with different temperature, 2, 25 and 45°C. Absorbances were measured from day 0 until day 15 and absorbance values were evaluated. All EGCG concentrations were measured using HPLC. Group 1 and 2 were taken to antibacterial activity test to observe the inhibition against *S. epidermidis* ATCC 35984 with 1% until 6% concentration. Ruler and scale measure was used to measure zone of inhibition. Different zone of inhibition were analyzed with t-independent test.

## RESULT AND DISCUSSION

**Buffer test:** The result showed that the most stable sample until 15 days was the extract with pH 4 buffer solution adding which performed stable absorbance during 15 days at 2°C temperature. However, there was no significant absorbance increased at 25°C if compared with storage condition of 45°C. It can be concluded that EGCG is stable in cold, while unstable in hot temperature. The absorbance value of extract with phosphoric acid adding increased significantly after 1 day at 2°C, 25°C or even 45°C. This showed the EGCG in the extract is unstable because it experienced polymerization and oxidation. Besides that, the absorbance value of extract without any buffer adding increased significantly after 1 day at 2, 25°C or even 45°C (Fig. 1).

In Table 1, EGCG concentration without buffer adding was 26.18  $\mu\text{g mL}^{-1}$ , while with phosphoric acid adjustment was 53.04  $\mu\text{g mL}^{-1}$  and twice bigger than no buffer adding. In otherwise, EGCG concentration in pH adjustment with pH 4 buffer solution was 60.98  $\mu\text{g mL}^{-1}$ . This study showed not only phosphoric acid buffer that could improve EGCG concentration and had better effectiveness than phosphoric acid. Cold extreme treatment and pH 4 buffer solution adding had the greatest concentration.

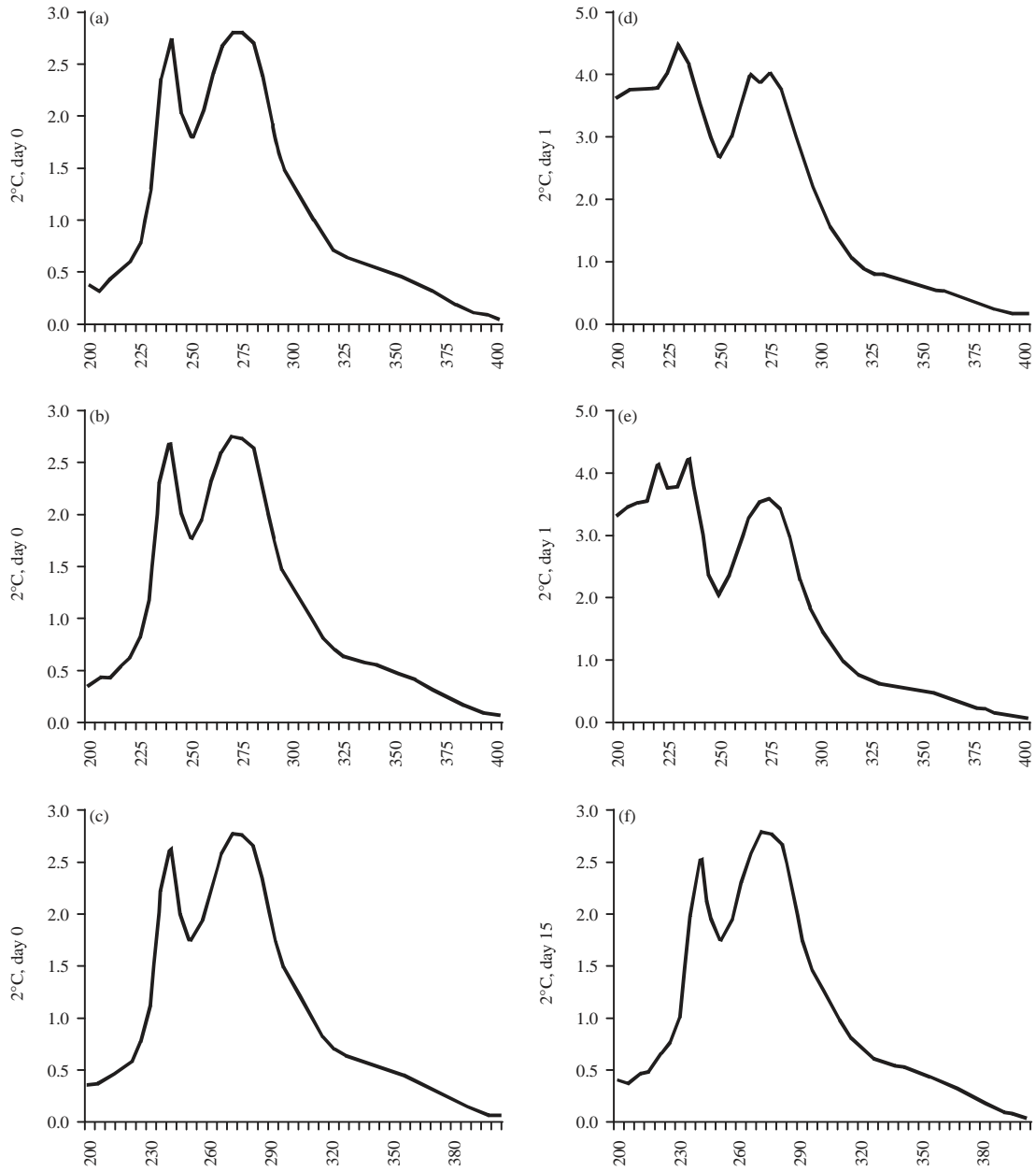


Fig. 1(a-f): Curve of absorbance, (a) Extract without the added of buffer days 0, (b) Extract with the added of phosphate acid pH 4 days 0, (c) Extract with the added of buffer solution pH 4 days 0, (d) Extract without the added of buffer days 1, (e) Extract with the added phosphoric acid pH 4 days 1 and (f) Extract with the added of buffer solution pH 4 day 15

Table 1: Ethyl acetate extract fraction of green tea leaves extract with phosphoric acid and pH 4 buffer measurement result

Replication	Concentration EGCG without buffer adding (w/w %)	Concentration EGCG with phosphoric acid adding pH 4 (w/w %)	Concentration EGCG with pH 4 buffer solution (w/w %)
1	25.79	50.51	61.38
2	26.57	55.57	60.58
Mean	26.18	53.04	60.98
SD	±0.78	±5.06	±0.80

Table 2: Zones of inhibition results zones of antibacterial inhibitions (mm) in100  $\mu$ L sample  $\pm$ SD in *Staphylococcus epidermidis* ATCC 35984

Replication	Adding of pH 4 buffer solution (%)						Adding of pH 4 phosphate acid (%)					
	1	2	3	4	5	6	1	2	3	4	5	6
1	13	16	20	22.0	24	25.0	25.0	28.0	32	33	35	36
2	13	16	20	22.0	24	26.0	25.0	29.0	32	33	35	36
3	13	16	20	23.0	24	26.0	26.0	28.0	32	33	35	36
Mean	13	16	20	22.3	24	25.6	25.3	28.3	32	33	35	36
SD	0	0	0	0.3	0	0.6	0.57	0.57	0	0	0	0

1: Fraction of ethyl acetate extract of green tea leaves concentration of 1%, 2: Fraction of ethyl acetate extract of green tea leaves concentration of 2%, 3: Fraction of ethyl acetate extract of green tea leaves concentration of 3%, 4: Fraction of ethyl acetate extract of green tea leaves concentration of 4%, 5: Fraction of ethyl acetate extract of green tea leaves concentrations of 5%, 6: Fraction of ethyl acetate extract of green tea leaves concentration of 6%

Table 3: Antibacterial activity of phosphate acid and buffer solution pH 4 in *Staphylococcus epidermidis* ATCC 35984

No	Groups name	Zones of inhibitions (mm)
1	Phosphoric acid	75
2	pH 4 buffer solution	25

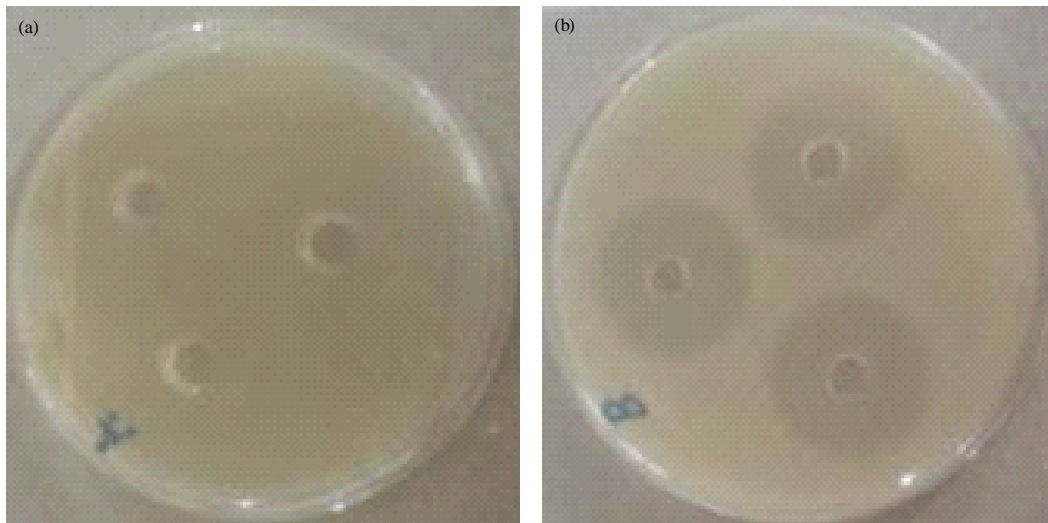


Fig. 2(a-b): Antibacterial activity of (a) Phosphoric acid and (b) pH 4 buffer solution in *Staphylococcus epidermidis* ATCC 35984

### Antibacterial activity test

**Antibacterial activity test in *Staphylococcus epidermidis* ATCC 35984 with buffer solution pH 4 and phosphoric acid pH 4:** In Table 2, ethyl acetate fraction of green tea extract zones of inhibition with phosphoric acid adding significantly larger than those created by the pH 4 buffer solution (group 2). This result showed there was a difference in zones of inhibition from 1-6% concentration in group 1 and 2.

Ethyl acetate fraction of green tea leaves extract with pH 4 phosphoric acid adding (group 1) was lower than those created by pH 4 buffer solution adding (group 2). On the other hand, ethyl acetate fraction of green tea leaves extract with pH 4 phosphoric acid adding produced larger zones of inhibition because phosphoric acid was had the greatest antibacterial activity than pH 4 buffer (Table 3). This was proved by phosphoric acid and pH 4 buffer antibacterial test in *S. epidermidis*

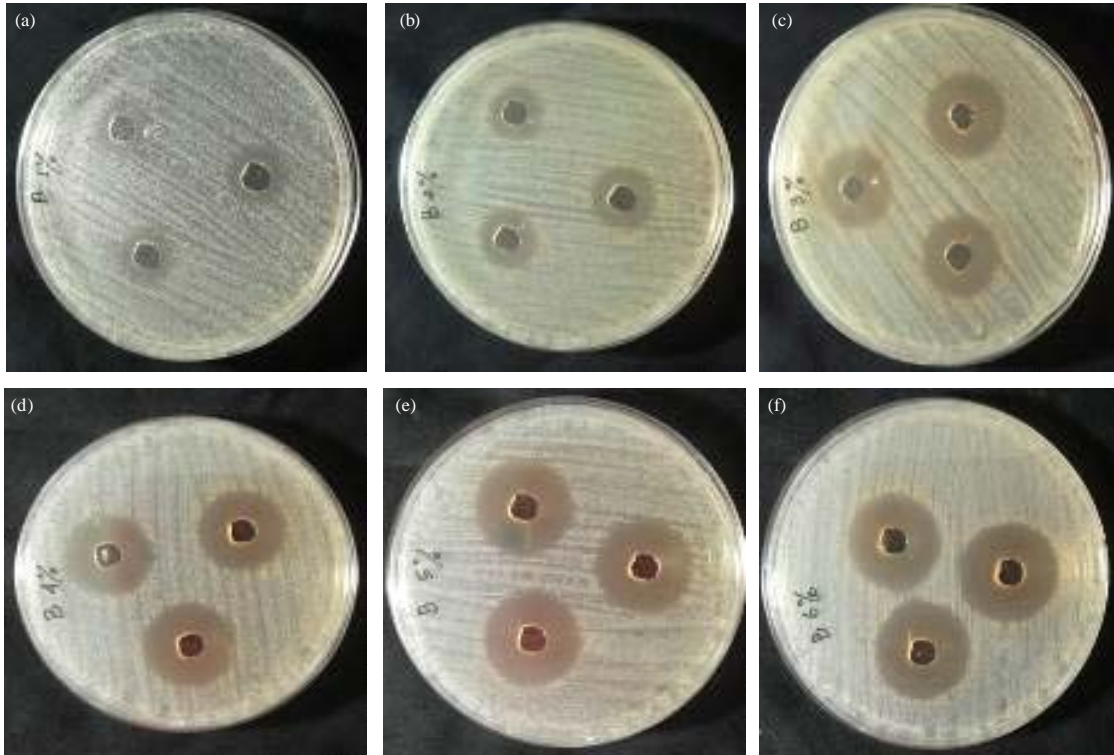


Fig. 3(a-f): *Staphylococcus epidermidis* inhibition of (a) Concentration of 1%, (b) A concentration of 2%, (c) Concentration of 3%, (d) Concentration of 4%, (e) Concentration of 5% and (f) Concentration of 6% with pH 4 buffer solution

bacteria. The result showed strong antibacterial activity that could be seen in zones of inhibition in all plate (Fig. 2) and result antibacterial test extract with pH 4 buffer solution and phosphoric acid in *S. epidermidis* could be seen in Fig. 3 and 4.

According to antibacterial activity of phosphoric acid that much greater than pH 4 buffer solution, therefore zones of inhibition group 1 was affected with phosphoric acid antibacterial activity so pH 4 buffer solution adding in extract was much better than phosphoric acid.

**Antibacterial activity test from ethyl acetate fraction of green tea leaves extract with buffer solution pH 4 methods in *Propionibacterium acnes* ATCC 6919:** *Propionibacterium acnes* ATCC 6919 is a Gram-positive bacterium which is a facultative anaerobic bacteria that cause acne specific (Bek-Thomsen *et al.*, 2008).

The research from Stratton *et al.* (2000) stated Epigallocatechin gallate concentration of 10% could cause erythema in test animals after 5 days and 7% concentration could cause erythema on day 15, while concentrations below did not cause toxicity within 30 days. So the antibacterial activity testing for ethyl acetate fraction of green tea leaves extract against *P. acnes* bacteria was conducted by making a series sample concentrations sample, consisted of 6 concentration namely 1, 2, 3, 4, 5 and 6% or concentrations of the sample used were 10, 20, 30, 40, 50 and 60 mg mL<sup>-1</sup>. This research was conducted by 3x replications in bacteria *P. acnes* ATCC 6919 at a concentration of 1-6%. Diameter measured was the average of the number of measurements 3x inhibition zone.

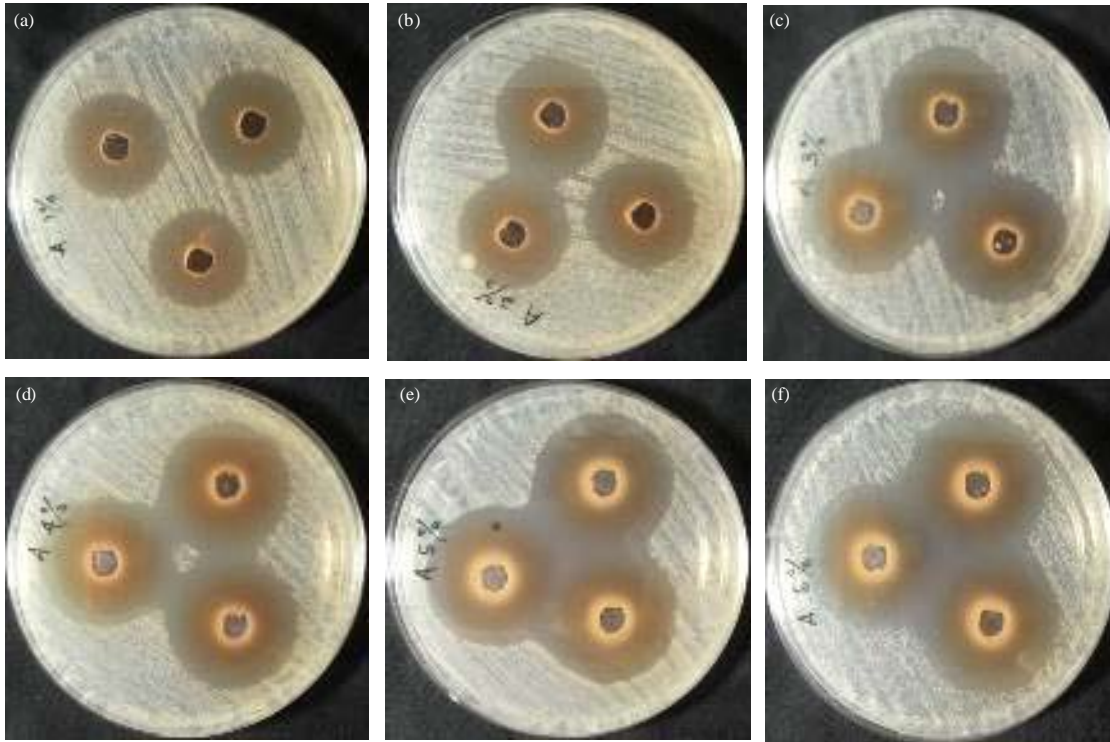


Fig. 4(a-f): *Staphylococcus epidermidis* inhibition of (a) Concentration of 1%, (b) A concentration of 2%, (c) Concentration of 3%, (d) Concentration of 4%, (e) Concentration of 5% and (f) Concentration of 6% with pH 4 phosphoric acid

Table 4: Zones of inhibition results zones of antibacterial inhibitions (mm) in 100  $\mu$ L sample  $\pm$ SD

Replication	<i>Propionibacterium acnes</i> ATCC 6919 (%)					
	1	2	3	4	5	6
1	23	26	29	32	34	38
2	21	25	30	33	35	38
3	20	23	30	33	36	39
Mean $\pm$ SD	21.3 $\pm$ 1.5	24.6 $\pm$ 1.5	29.6 $\pm$ 0.5	32.6 $\pm$ 0.5	35 $\pm$ 1	38.3 $\pm$ 0.5

The results diameter zone of inhibition on *P. acnes* ATCC 6919 was presented in Table 4 and result antibacterial test extract with buffer solution pH 4 in *P. acnes* could be seen in Fig. 5.

From Table 4, it can be concluded that the greater the concentration of ethyl acetate fraction of green tea leaves extract, the greater its inhibitions against *P. acnes*. Concentration of 6% had the greatest power resistor that can be used as a dose of anti acne. When compared with the research of Bashir *et al.* (2014), green tea extract using methanol from Indonesia had antibacterial activity against *K. pneumoniae*, *S. pyogenes*, *S. epidermidis*, *S. aureus*, *S. marcesscens*, *P. aeruginosa* and *E. coli*, with large barriers for Indonesian are 3.33 $\pm$ 1.52, 5.0 $\pm$ 0.0, 5.33 $\pm$ 2.51, 0.0 $\pm$ 0.0, 9 $\pm$ 4.58, 1.33 $\pm$ 1.15 and 1.0 $\pm$ 0.0. This study showed the ethyl acetate fraction of green tea leaves extract had antibacterial activity that higher than previous studies. The ethyl acetate fraction of green tea leaves extract with EGCG concentration of 60.98% w/w could strongly kill *P. acnes* and *S. epidermidis*.



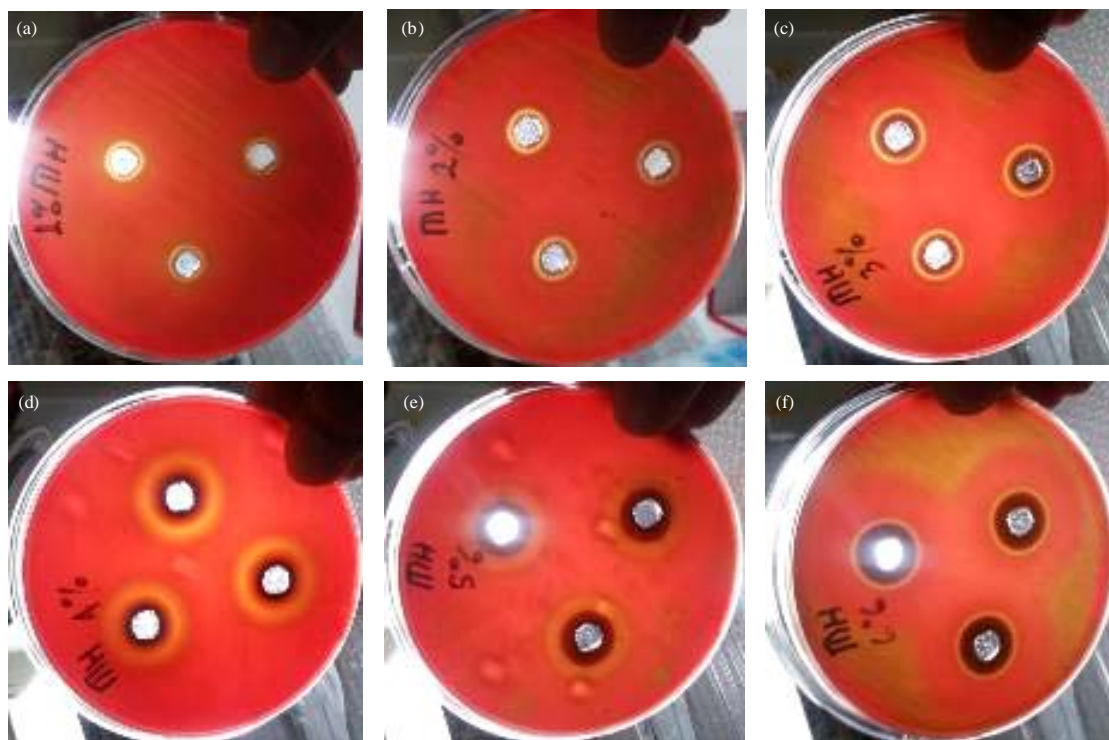


Fig. 5(a-f): *Propionibacterium acnes* inhibition of (a) Concentration of 1%, (b) A concentration of 2%, (c) Concentration of 3%, (d) Concentration of 4%, (e) Concentration of 5% and (f) Concentration of 6% with pH 4 buffer solution

## CONCLUSION

The extreme cold temperature treatment and pH 4 buffer solution adding at storage condition of 2°C gave the most stable absorbance value during 15 days and the highest EGCG concentration namely 60, 98%. The antibacterial activity of ethyl acetate fraction of green tea leaves extract with phosphoric acid adding had a higher inhibition ability than the extract with pH 4 buffer solution adding. It was caused by antibacterial activity from phosphoric acid so pH 4 buffer solution adding in green tea leaves extraction process with extreme cold temperature treatment is the best method. Ethyl acetate fraction of green tea leaves extract concentration of 1% had a strong antibacterial activity in inhibiting the growth of *P. acnes* followed by a greater concentration up to 6%, this showed that concentration of 6% can be used as an anti acne dosage.

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