CHAPTER III

RESULTS

1. Polysaccharide Gel (PG) from Fruit-Hulls of Durian

Polysaccharide Gel (PG) was isolated from dried fruit-hulls of durian and purified by the method modified by Pongsamart and Panmauang (1998). The dried crude extract of polysaccharide gel (PG) was ground to powder. The polysaccharide gel (PG) crude extract was further purified and pulverized to fine powder and then passed through 60 mesh sieve. The creamy white powder was obtained. The pH value and apparent viscosity of PG gel at 3% concentration was 2.27±0.01 and 439.67±0.58 cps, respectively. The final yield of polysaccharide gel (PG) was 6.5%. The PG gel dried powder is shown in Figure 14.

2. Physical properties of Polysaccharide Gel (PG)

2.1 pH

The polysaccharide gel (PG) solutions were prepared at concentrations of 1-6% w/v PG. The pH was measured by using pH meter (Mettler Toledo MP230). The pH was measured repeatedly three times. The mean values of three measurements were calculated. The profile of pH mean values versus concentrations of PG is shown in Figure 15. It was noticed that a decreasing of pH was respected to an increasing of concentration of PG. The pH values were slightly decreased at the higher concentration of PG.

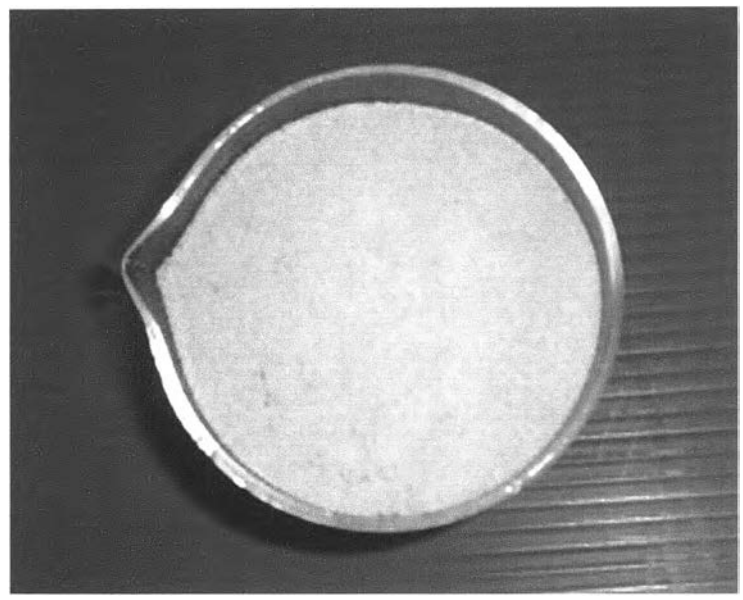


Figure 14. Polysaccharide gel (PG) isolated from dried fruit-hulls of durian.

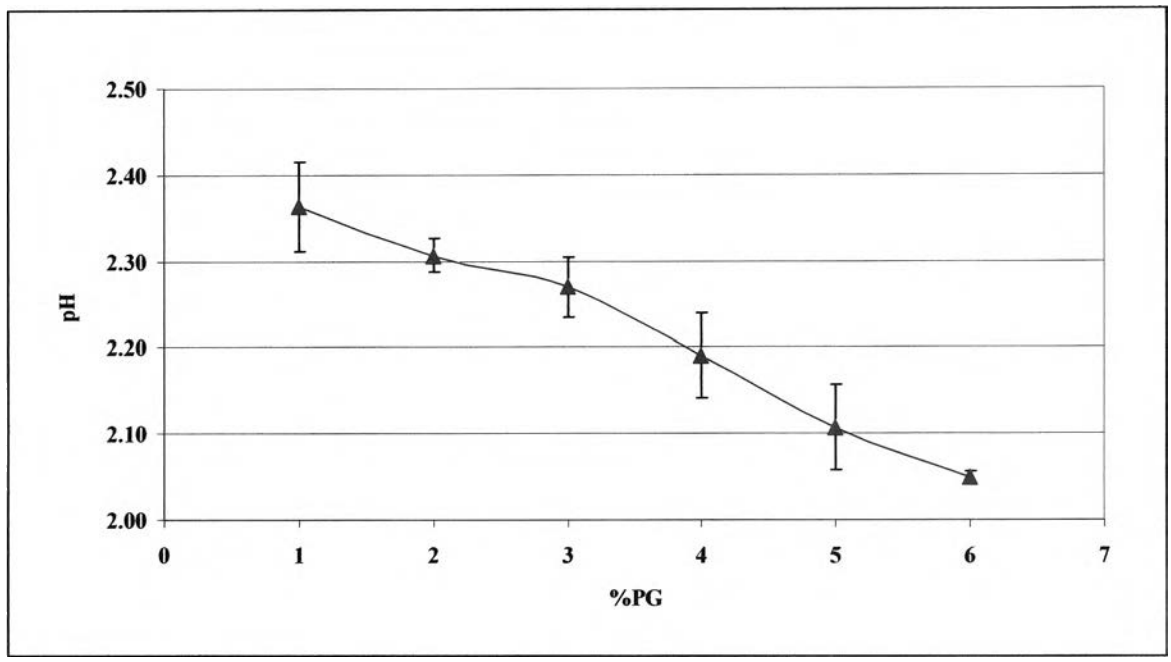


Figure 15. The pH profile of polysaccharide gel (PG) at different concentrations Data are means±SD

2.2 Viscosity

The PG solutions of various concentrations at 1-6% w/v PG were prepared. The viscosity at each concentration was measured by Brookfield Viscometer (model LVDV-I+). The results of the mean values were calculated from three determinations. The apparent viscosity (mean±SD) of PG solutions was 81.13±1.17, 182.60±1.49, 439.67±0.58, 949.07±1.01, 4757.67±2.52 and 9843.00±2.00 cps, respectively. The data are shown in Figure 16. It was showed that the viscosity was slightly increased at low concentration of PG, whereas the concentration at more than 4% PG the viscosity was rapidly increased.

3. Compatibility studies of Polysaccharide Gel (PG)

3.1 Acid and Base

Compatibility studies of PG with acid and base were performed by using hydrochloric acid and sodium hydroxide. The 3% PG solution was added with a volume of 5M hydrochloric acid (HCl) or 5M sodium hydroxide (NaOH) to make PG solutions reach the different final acid or basic pH values, respectively. The viscosity of PG solutions was measured at pH values range from 1.04 to 10.16. The data reported were the mean values of three determinations as illustrated in Figure 17. The viscosity of PG was gradually decrease with respected to the increasing of pH. Acid pH lower than 2 had less effect on viscosity of PG.

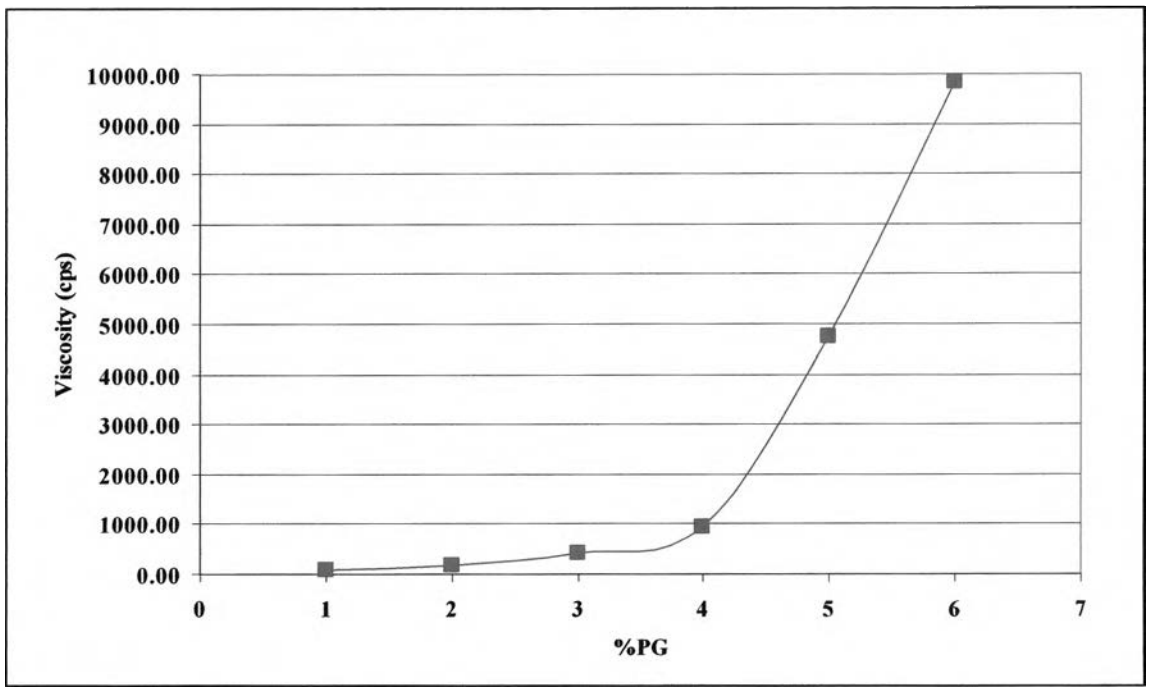


Figure 16. Viscosity profile of polysaccharide gel (PG) at different concentrations

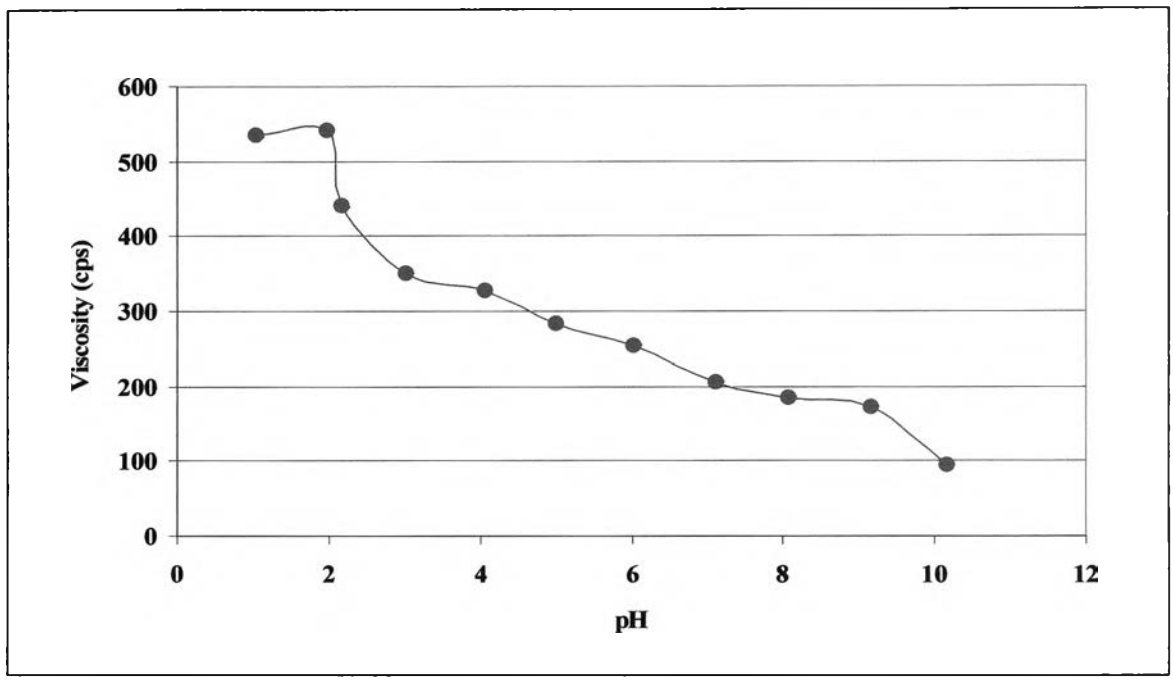


Figure 17. Effect of acid (HCl) and base (NaOH) on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water

3.2 Divalent cations

Solutions of divalent catiions such as $CaCl_2$, $MgCl_2$, $ZnSO_4$ and $FeSO_4$ were prepared at concentration of 1M. Each of solution was added to 3% PG solution in order to make a final concentration of 0.02, 0.04, 0.06, 0.08 and 0.1M, respectively. The results of divalent catiions eletrolytes on the viscosity of PG are demonstrated in Figure 18. All of tested divalent cations markedly affected to increase the viscosity of PG solution. $CaCl_2$ and $MgCl_2$ gradually increased the viscosity of 3% PG, $FeSO_4$ at concentration more than 0.08 M showed rapidly increasing PG viscosity, whereas $ZnSO_4$ showed rapidly and highly increasing PG viscosity even at very low concentration.

3.3 Organic solvents

Effect of organic solvent on viscosity of PG was studied by adding to the 3% PG solutions with organic solvents such as ethyl alcohol and isopropyl alcohol to make concentrations at 5, 10, 15, 20, 25 and 30% w/v of solvent and homogenously mixed. The effect of organic solvents on the viscosity of polysaccharide gel (PG) is shown in Figure 19. The viscosity of PG increased slightly as the solvent concentration increased up to 15% w/v with both ethyl alcohol and isopropyl alcohol, but at concentration of the solvents higher than 15% the viscosity of PG increased rapidly. Isopropyl alcohol at 30% concentration gave the high viscosity of PG to 9840.33$\pm$1.53 cps. In addition ethyl alcohol at 30% concentration gave viscosity of PG to 9830$\pm$2.00 cps. Precipitation of PG was formed at the concentration of these solvents higher than 40% w/v.

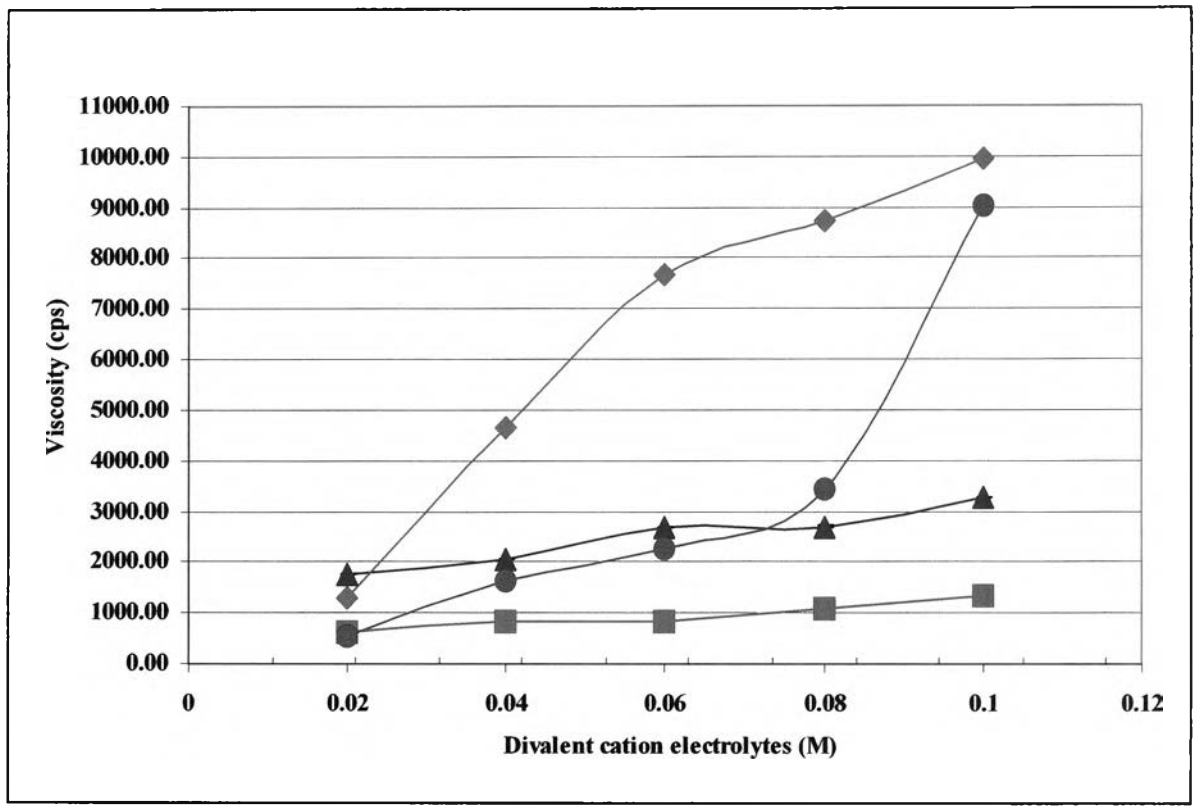


Figure 18. Effect of eletrolytes of divalent cations on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water -▲- $CaCl_2$; -■- $MgCl_2$; -●- $FeSO_4$; -◆- $ZnSO_4$

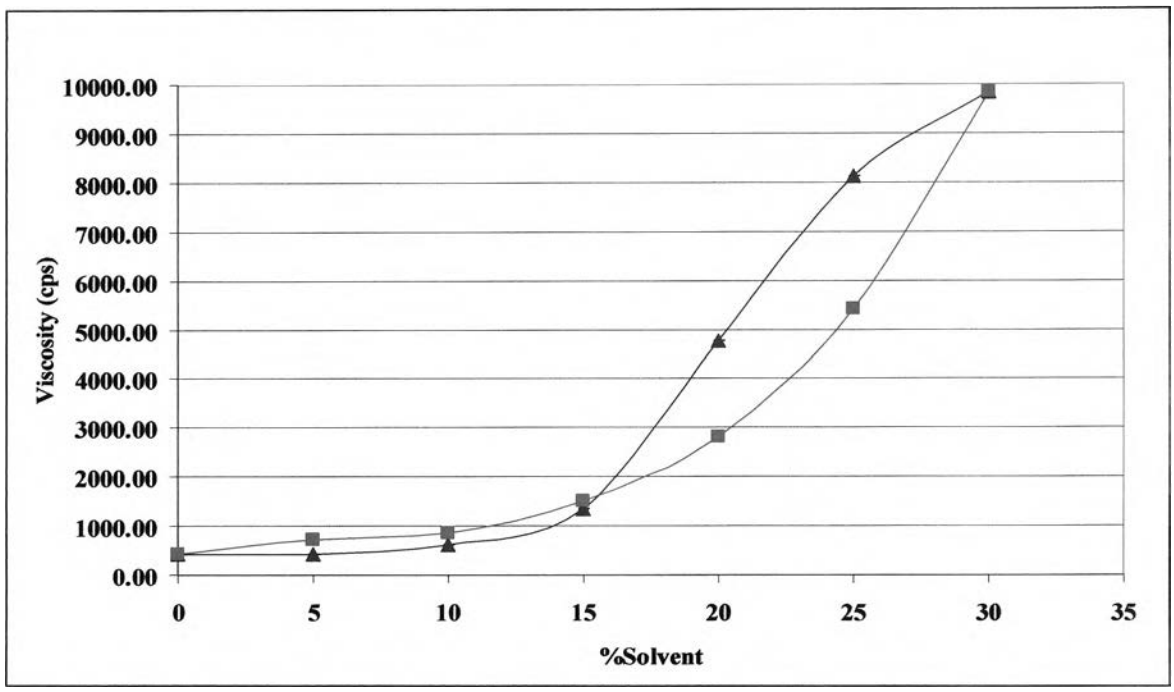


Figure 19. Effect of organic solvents on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water -▲- Ethyl alcohol; -■- Isopropyl alcohol

3.4 Humectants

The different types of humectants such as propylene glycol, glycerin and sorbitol were studied by adding each of humectant to 3% PG solution to make a final concentration at 5 to 30% w/v of humectant. The results in Figure 20 showed that as the concentrations of propylene glycol, glycerin and sorbitol increased the apparent viscosity of PG increased accordingly. Propylene glycol gave the highest effect on viscosity values range from 648.17±1.26 to 2679.67±1.53 cps. In addition the viscosities of PG effected by glycerin and sorbitol at 5 to 30% w/v were 468.43±1.56 to 1041.33±2.08 cps and 465.77±1.97 to 931.97±2.05 cps, respectively. All tested humectanats showed low effect to the apparent viscosity of PG at the concentration used in PG gel preparation in this study.

3.5 Emollient

Amerchol L101 was used in this study by adding an appropriate volume of amerchol L101 to the 3% PG solution and homogeneously mixed to make 5, 10, 15, 20, 25 and 30% w/v concentrations of amerchol L101. The viscosities (mean±SD) of PG mixture were 439.67±0.58, 532.27±2.05, 557.77±1.10, 645.33±1.53, 1114.00±2.00, 1740±1.53 and 1774±2.00 cps, respectively. The viscosity profile illustrated in Figure 21. It was showed that the viscosity of PG solution increased with increasing concentration of amerchol L101.

3.6 Preservative

An aqueous solution of PG at 3% concentration was added with paraben concentrate and mixed to make different concentrations from 0.2 to 5% w/v of paraben concentrate. Effect of preservative to viscosity of PG solution was shown in Figure 22. Results showed that preservative showed less effect to increase viscosity of PG at concentration used in the formulation of PG preparation which was only at 1% w/v. The apparent viscosities of PG solutions with 1-5% w/v paraben concentrate were 441.40±0.78 to 474.73±0.64 cps.

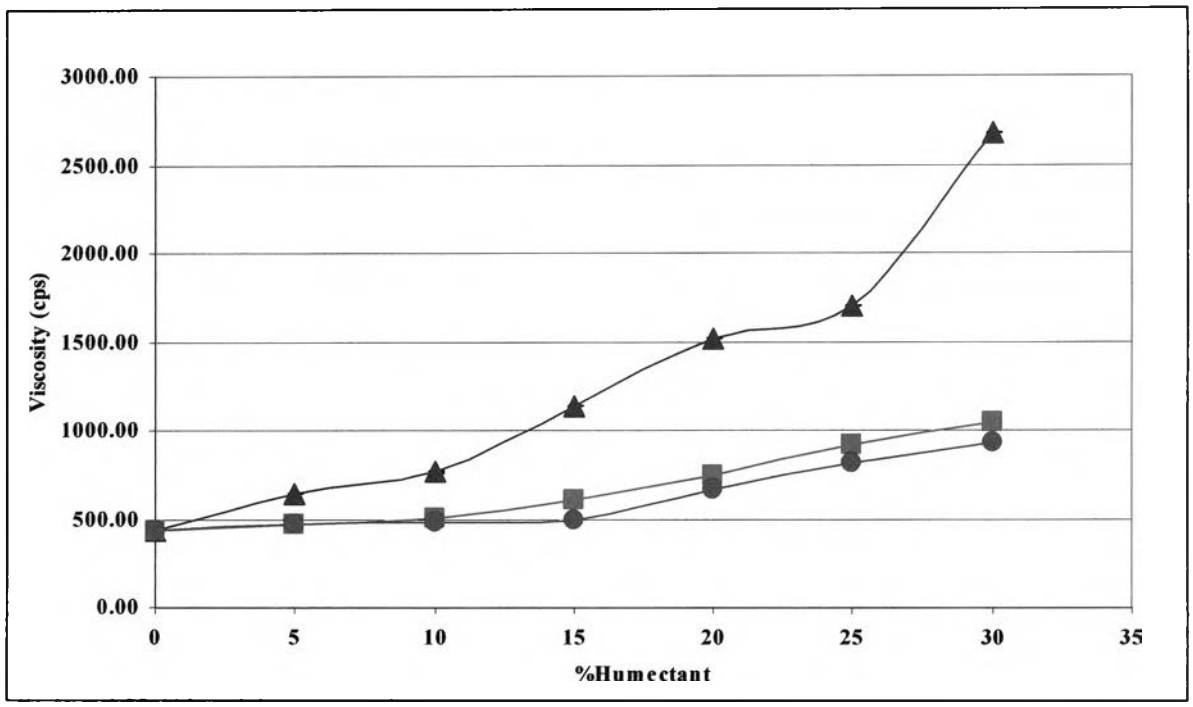


Figure 20. Effect of humectants on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water - ▲ - Propylene glycol; - ■ - Glycerine; - ● - Sorbitol

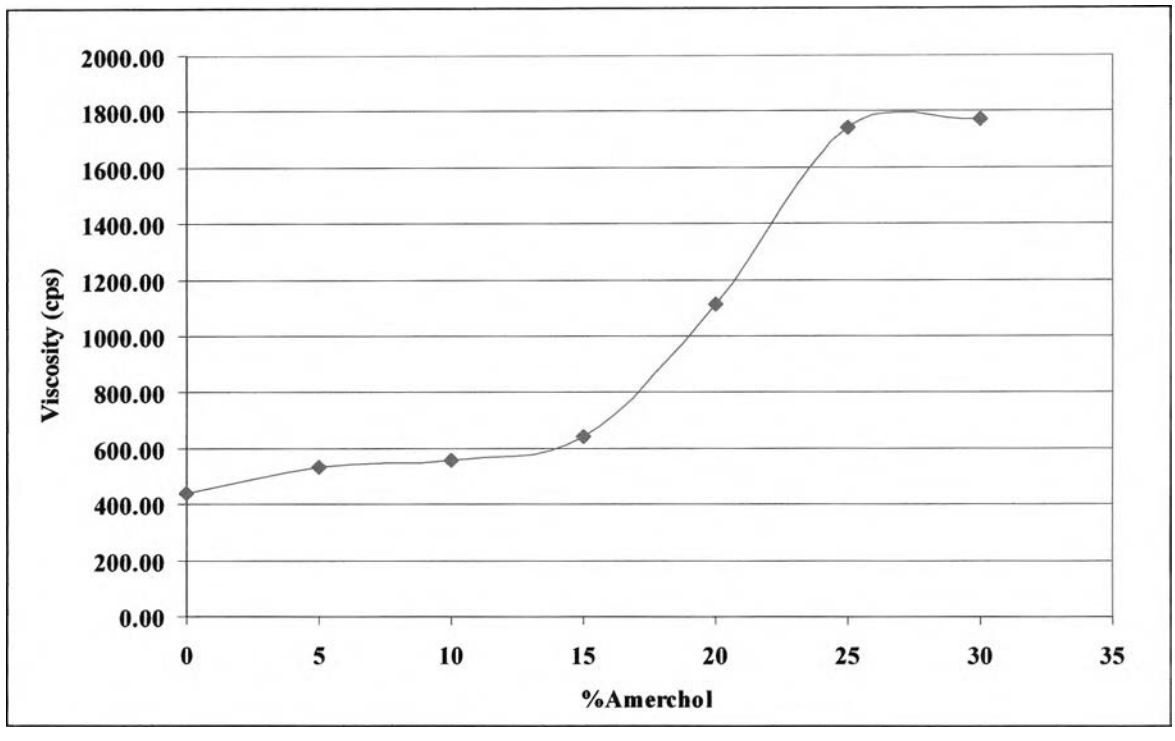


Figure 21. Effect of amerchol L101 on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water

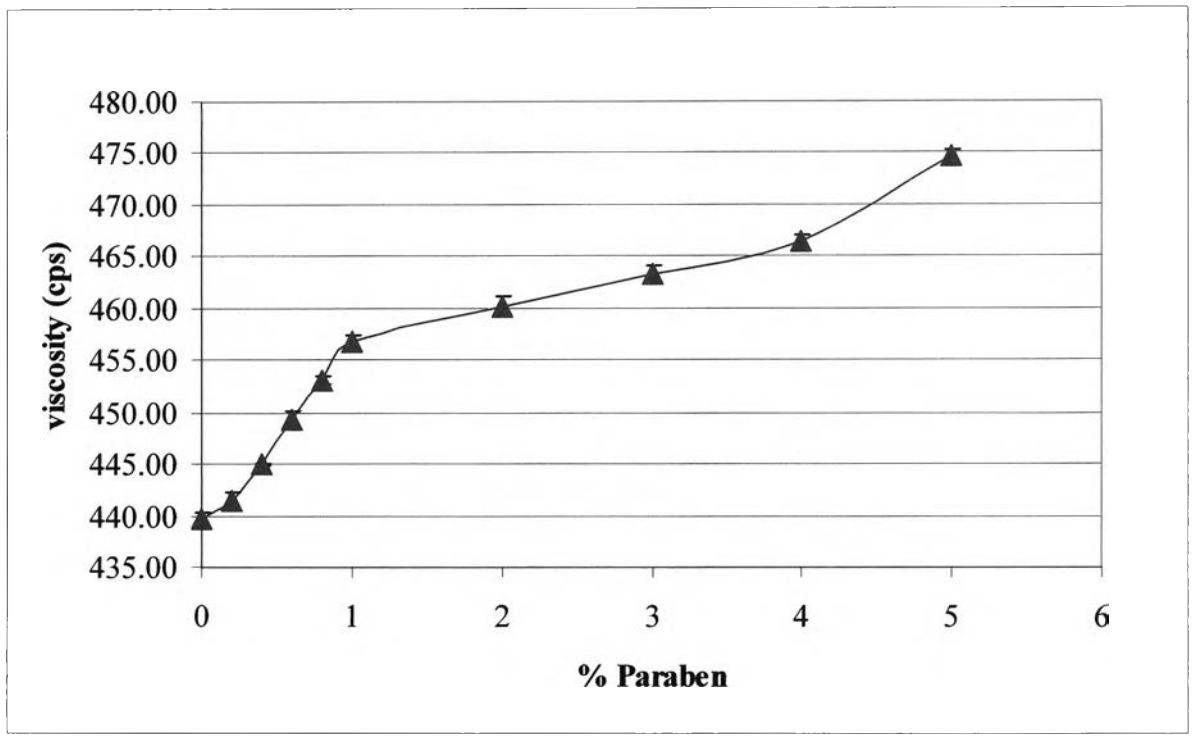


Figure 22. Effect of paraben concentrate on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water

4. Effect of buffers on polysaccharide gel (PG)

The stock solutions of citrate buffer at pH 4, 4.5, 5, 5.5 and 6; and phosphate buffer at pH 5.5, 6, 6.5, 7 and 7.5 were prepared and PG was added to make 3% PG in each buffer solution. The influence of citrate buffer and phosphate buffer of different pHs on the apparent viscosity is shown in Figure 23. The result showed that citrate buffer at any pH values gave less effect on viscosity of PG. In contrast phosphate buffer effected to increase apparent viscosity of PG, especially at the higher pH values of pH6. The viscosity of PG in citrate buffer were between 357.40±2.05 cps to 210.83±1.72 cps whereas the viscosity values in phosphate buffer were 910.57±1.86 to 9839.33±2.08 cps.

5. Determination of free radical scavenging activity of curcuminoid by using DPPH method

The H-donor activity of various samples was measured by 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Abe *et al.*, 1998; Parejo *et al.*, 2002). The DPPH is a stable radical, its methanolic solution shows an absorbency maximum at 517 nm. The H-donor molecules reduce DPPH radical and thus decrease its absorbance at 517 nm. The polar subfraction of the methanol extract of intact sample and methanol extract of various samples was able to reduce the stable free radical DPPH to the yellow-colored diphenylpicrylhydrazine.

The IC_{50} values of DPPH radical scavenging activities of various samples are given in Table 4. Gallic acid as a stand was used in all parallel experiments and a logarithmic curve of gallic acid was plotted and used to calculate the value of IC_{50} was demonstrated in Figure 24. The IC_{50} value was calculated by a logarithmic regression curve. The data demonstrated that the ability of tested samples in scavenging DPPH radicals measured as IC_{50} value varied form 14.81 to 54.72 mg/ml. The curcuminoid which obtained from Thai-china flavours and fragrances industry CO., LTD, gave the highest activity with an IC_{50} value of 14.81 mg/ml in comparision with the synthetic antioxidant gallic acid that gave IC_{50} value of 4.39 mg/ml. The betel vine oil and oleoresin of curcumin showed less or no DPPH radical scavenging activity.

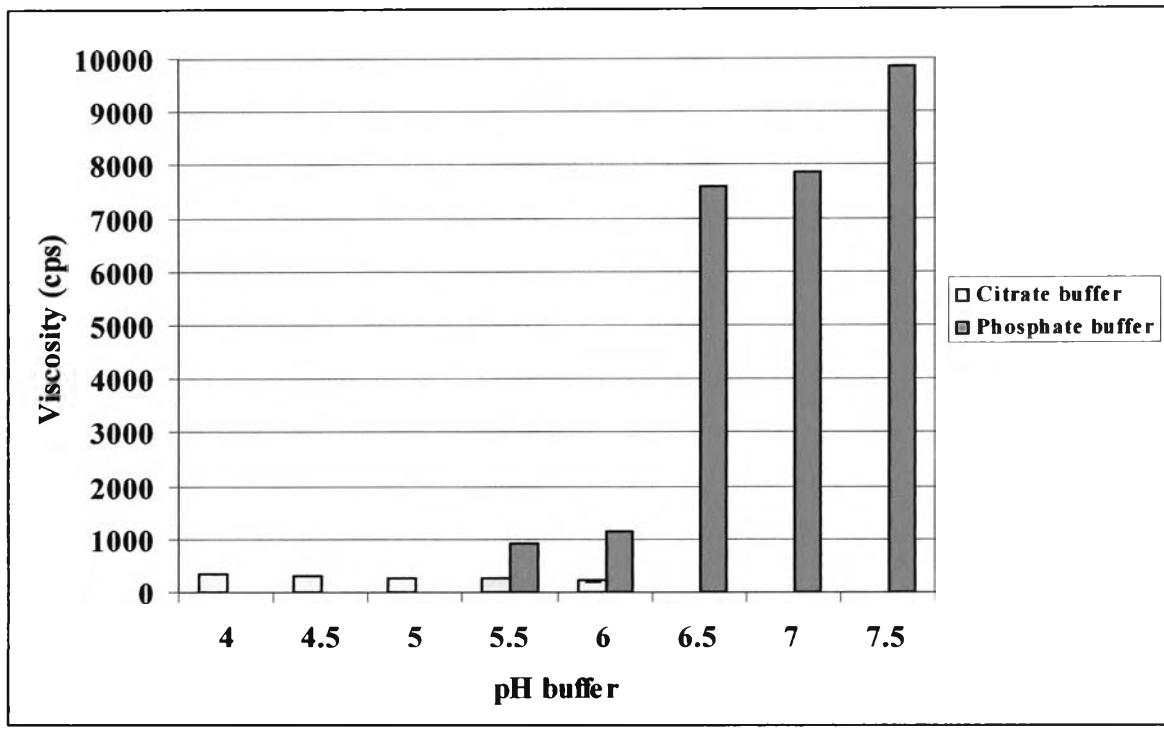


Figure 23. Effect of buffers on the apparent viscosity of 3% w/v polysaccharide gel (PG) in distilled water

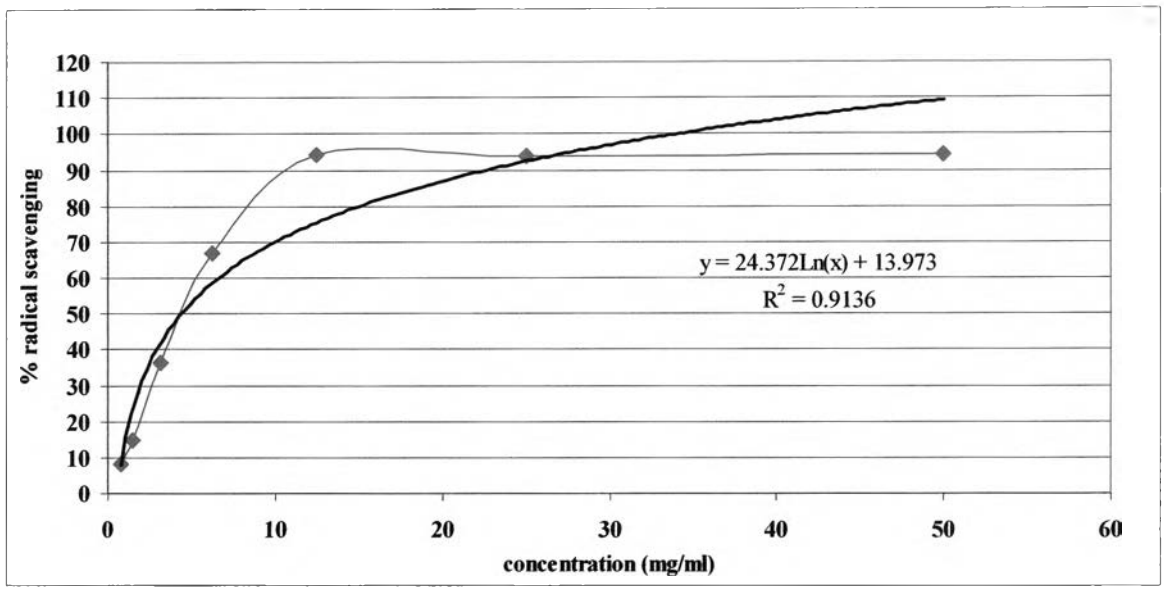


Figure 24. A logarithmic regression curve of gallic acid standard

Table 4. IC_{50} values of DPPH radical scavenging activities in various samples

Samples	DPPH scavenging activity IC_{50} (mg/ml)
Gallic acid (standard)	4.39
Betel vine oil	54.72
Oleoresin	55.58
Curcuminoid[a]	35.19
Curcuminoid[b]	14.81

a- sample from The Government Pharmaceutical Organization

b- sample from Thai-china flavours and fragrances industry CO., LTD

6. Antimicrobial susceptibility test of betel vine oil

Betel vine showed inhibitory activity against bacteria and fungus by agar diffusion and broth macrodilution method. The nine bacterial strains, *Staphylococcus aureus* and *Staphylococcus epidermidis* are normal flora which cause skin infection and pus, *Micrococcus luteus* and *Bacillus subtilis* were found in skin as normal flora and in environment; represent a test gram positive bacteria; *Escherichia coli and Proteus vulgaris* can found in gastrointestinal tract as normal flora, *Salmonella typhimurium* cause of food poisoning, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa* cause infection in immunocompromised individuals; represent a test gram negative bacteria; two yeast strains, *Candida albicans* cause infection in healthy individuals, *Saccharomyces cerevisiae* found in the environment; acnes causing bacteria *Propionibacterium acnes*.

6.1 Agar diffusion

The results of inhibition zone diameters of microbial growth inhibition on agar plates of betel vine oils demonstrated in Table 5-7. The mean (SD) values of inhibition zones were obtained at the lowest concentration 0.08% v/v of betel vine oil against bacteria, *Micrococcus luteus, Bacillus subtilis, Proteus vulgaris, Pseudomonas aeruginosa* and fungus, *Saccharomyces cerevisiae*, were 12.53 (1.84), 10.20 (0.10), 10.27 (0.25), 10.53 (0.45) and 10.20 (0.20) mm, respectively. Diameter of microbial inhibition zone against fungus, *Candida albicans* was 11.20 (0.26) mm with 0.16% v/v concentration of the betel vine oil. Inhibition zones obtained at 0.31% v/v betel vine oil against *Propionibacterium acnes, Staphylococcus aureus, Staphylococcus epidermidis*, *Escherichia coli*, *Salmonella typhimurium* and *Klebsiella pneumoniae* were 9.67 (0.58), 10.33 (0.58), 10.17 (0.29), 10.85 (0.68), 11.28 (0.85) and 10.98 (0.53) mm, respectively. In Figure 25 illustrated the inhibition zones of various concentrations (%) of betel vine oil were between 17.00 (0.50) to 10.33 (0.58) mm against *Staphylococcus aureus*. Figure 26 showed the inhibition zones of various concentrations (%) of betel vine oil against *Propionibacterium acnes* were between 16.03 (0.25) to 9.67 (0.58) mm.

The results of antimicrobial activity of curcuminoid and oleoresin were showed in Table 8. The inhibition zones of curcuminoid in 20% MeOH were in the range of 9.80 (0.25) to 10.87 (0.65) mm in diameter against *Staphylococcus aureus* and *Staphylococcus epidermidis*. In addition, the inhibition zones of oleoresin in 20% MeOH were 11.70 (0.58) to 13.00 (0.50) mm in diameter against *Staphylococcus aureus* and *Staphylococcus epidermidis*. Whereas the control (20% MeOH) showed no inhibition zone.

The inhibition zones of oleoresin in 10% DMSO were also determined as demonstrated in Table 9 that the clear zones of 10.33 (0.58) to 11.40 (0.06) mm in diameter against *Staphylococcus aureus* and *Staphylococcus epidermidis* were obtained.

6.2 Broth macrodilution test

The results of antimicrobial activity in Table 10 showed MIC and MBC values of betel vine oil against 9 tested bacteria and 2 fungi. MBCs of betel oil were in ranges of 0.020 to 0.156%. The MIC and MBC values of betel vine oil were 0.098 and 0.020% (v/v), respectively against *Bacillus subtilis* and *Candida albicans*. MIC and MBC values of betel vine oil were 0.020 and 0.039% (v/v) against *Propionibacterium acnes, Micrococcus luteus, Proteus vulgaris, Pseudomonas aeruginosa* and *Saccharomyces cerevisiae,* repectively. MIC and MBC were 0.039 and 0.078% (v/v) against *Staphylococcus aureus, Staphylococcus epidermidis* and *Klebsiella pneumoniae.* MIC and MBC of betel vine oil were 0.078 and 0.156% (v/v) against *Salmonella typhimurium* and *Escherichia coli*, respectively. Figure 27 demonstrated the MIC value of betel vine oil at 0.039% (v/v) concentrations in MHB medium against *Staphylococcus epidermidis*, a clear solution of medium demonstrated no visible growth of bacterium at this lowest betel vine oil concentration. While Figure 28 illustrated that MIC of betel vine oil was at 0.020% (v/v) concentrations in BHIB medium against *Propionibacterium acnes*.

Table 5. Antibacterial activity of betel oil on growth of gram positive bacteria by agar diffusion method.

% betel oil in 0.1% tween 80	Diameter of inhibition zone, mm mean (SD)				
	P. acnes	*S. aureus*	*S. epidermidis*	*M. luteus*	*B. subtilis*
50	15.17 (0.29)	17.00 (0.50)	16.00 (2.00)	18.23 (0.83)	18.50 (0.40)
20	14.10 (0.17)	16.33 (0.58)	15.17 (1.26)	17.78 (1.13)	17.50 (0.87)
10	13.03 (0.15)	15.17 (1.53)	14.33 (1.44)	17.47 (1.19)	15.97 (0.75)
5	11.90 (0.10)	12.50 (0.50)	12.83 (0.58)	17.00 (1.32)	15.33 (1.26)
2.5	11.60 (0.36)	11.83 (0.58)	11.67 (0.29)	16.33 (1.26)	14.67 (1.04)
1.25	10.67 (0.29)	11.00 (0.87)	11.17 (0.29)	15.73 (1.07)	13.88 (1.30)
0.63	10.40 (0.10)	10.67 (0.76)	10.50 (0.50)	15.27 (1.17)	12.93 (1.40)
0.31	10.12 (0.13)	10.33 (0.58)	10.17 (0.29)	14.83 (1.26)	12.13 (1.48)
0.16	NZ	NZ	NZ	14.33 (1.17)	10.67 (0.47)
0.08	NZ	NZ	NZ	12.53 (1.84)	10.20 (0.10)
Control (0.1%tween)	NZ	NZ	NZ	NZ	NZ

Table 6. Antibacterial activity of betel oil on growth of gram negative bacteria by agar diffusion method.

% betel oil in 0.1% tween 80	Diameter of inhibition zone, mm mean (SD)				
	E. coli	*P. vulgaris*	*S. typhimurium*	*K. pneumoniae*	*Ps. aeruginosa*
50	14.40 (0.79)	18.00 (0.10)	15.00 (1.42)	15.47 (0.87)	18.07 (0.71)
20	13.90 (0.66)	16.40 (1.10)	14.67 (1.43)	14.97 (1.11)	17.60 (0.75)
10	13.62 (0.53)	15.43 (1.16)	14.17 (1.47)	14.63 (1.29)	16.83 (1.04)
5	13.13 (0.45)	15.20 (1.14)	13.60 (1.15)	14.03 (1.11)	16.07 (0.83)
2.5	12.57 (0.59)	14.10 (0.96)	12.80 (1.18)	13.57 (1.16)	15.33 (0.91)
1.25	12.18 (0.63)	12.77 (0.21)	12.30 (0.96)	12.73 (1.17)	14.43 (0.93)
0.63	11.47 (1.01)	11.87 (0.78)	11.95 (1.24)	12.10 (0.80)	13.57 (0.55)
0.31	10.85 (0.68)	11.30 (0.70)	11.28 (0.85)	10.98 (0.53)	12.53 (0.50)
0.16	NZ	10.67 (0.57)	NZ	NZ	11.40 (0.78)
0.08	NZ	10.27 (0.25)	NZ	NZ	10.53 (0.45)
Control (0.1%tween)	NZ	NZ	NZ	NZ	NZ

Table 7. Antibacterial activity of betel oil on growth of fungi by agar diffusion method.

% betel oil in 0.1% tween 80	Diameter of inhibition zone, mm mean (SD)	
	C. albican	*S. cerevisiae*
50	15.43 (0.57)	17.27 (0.25)
20	15.10 (0.20)	16.87 (0.35)
10	14.47 (1.12)	15.93 (0.12)
5	14.07 (0.87)	15.90 (0.90)
2.5	13.97 (0.47)	14.17 (0.76)
1.25	12.93 (0.35)	13.83 (0.76)
0.63	12.37 (0.38)	13.10 (0.26)
0.31	11.37 (0.55)	11.67 (0.83)
0.16	11.20 (0.26)	10.80 (0.26)
0.08	NZ	10.20 (0.20)
Control (0.1%tween)	NZ	NZ

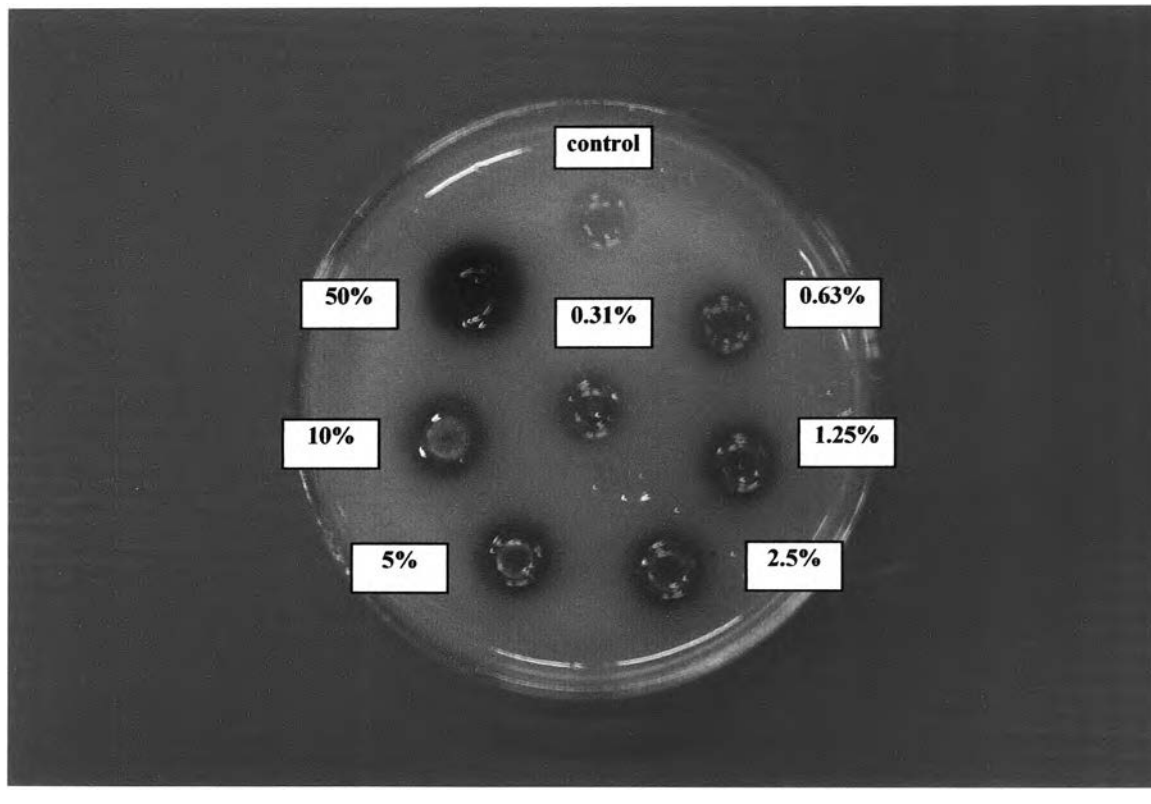


Figure 25. Microbiological assay plate for *S. aureus* ATCC 6538P on medium MHA at different concentrations (%) of betel vine oil in 0.1% tween 80 and control was 0.1% tween 80

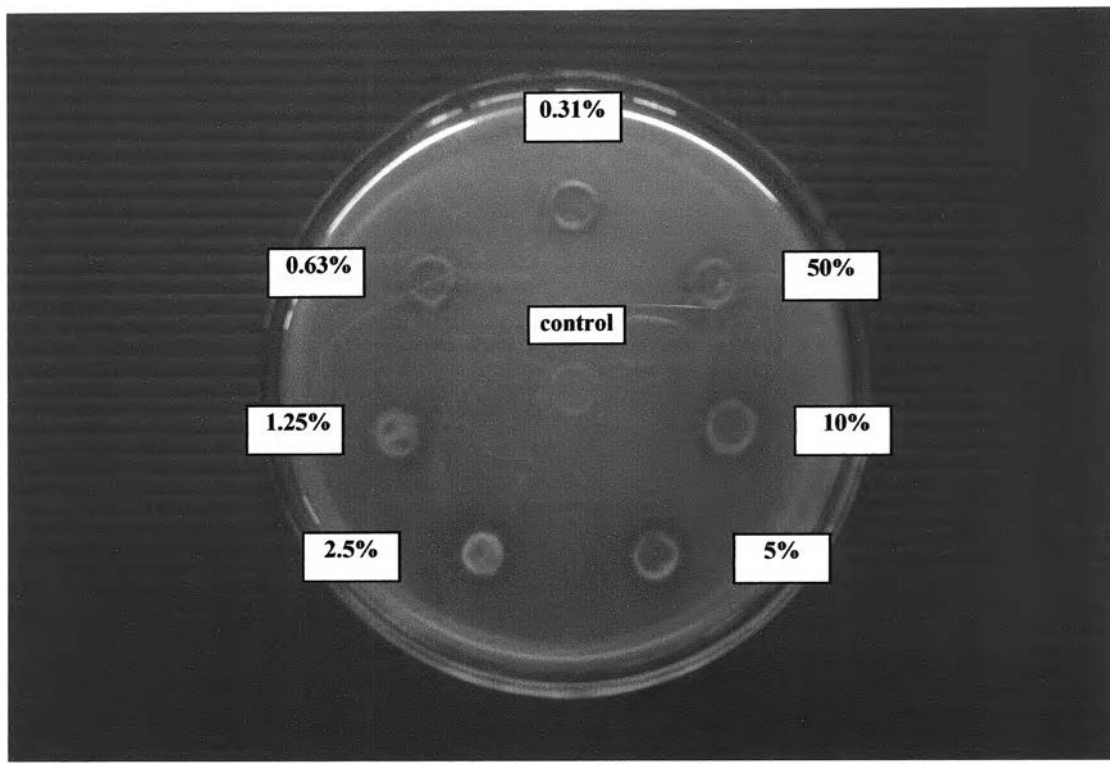


Figure 26. Microbiological assay plate for *Propionibacterium acnes* on medium BHIA. Cups contain different concentrations (%) of betel vine oil in 0.1% tween 80 was used as control.

Table 8. Antimicrobial activity of curcuminoid and oleoresin on growth of microorganisms by agar diffusion method. NZ = no inhibition zone

% Concentration in 20% methanol	Diameter of inhibition zone, mm mean (SD)	
	S. aureus	*S .epidermidis*
Curcuminoid 50	10.70 (0.69)	10.87 (0.65)
Curcuminoid 25	9.80 (0.25)	9.87 (0.15)
Oleoresin 50	12.60 (0.64)	13.00 (0.50)
Oleoresin 25	11.70 (0.58)	12.33 (0.58)
Control (20%methol)	NZ	NZ

Table 9. Antimicrobial activity of oleoresin on growth of microorganisms by agar diffusion method

% oleoresin in 10%DMSO	Diameter of inhibition zone, mm mean (SD)	
	S .aureus	*S. epidermidis*
5	11.23 (0.25)	11.40 (0.06)
2.5	10.72 (0.26)	10.67 (0.51)
1.25	10.50 (0.50)	10.63 (0.46)
0.63	10.33 (0.58)	10.59 (0.21)
Control(10%DMSO)	10.00 (0.89)	10.07 (0.86)

Table 10. Minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of betel vine oil against microorganisms by broth macrodilution method in MHB medium except for *P. acnes* was in BHB medium

Microorganisms	Betel vine oil	
	MIC (%v/v)	MBC (%v/v)
P. acnes	0.020	0.039
S. aureus	0.039	0.078
S. epidermidis	0.039	0.078
M. luteus	0.020	0.039
B. subtilis	0.010	0.020
E.coli	0.078	0.156
P. vulgaris	0.020	0.039
Salmonella typhimurium	0.078	0.156
K. pneumoniae	0.039	0.078
Ps. aeruginosa	0.020	0.039
C. albican	0.010	0.020
S. cerevisiae	0.020	0.039

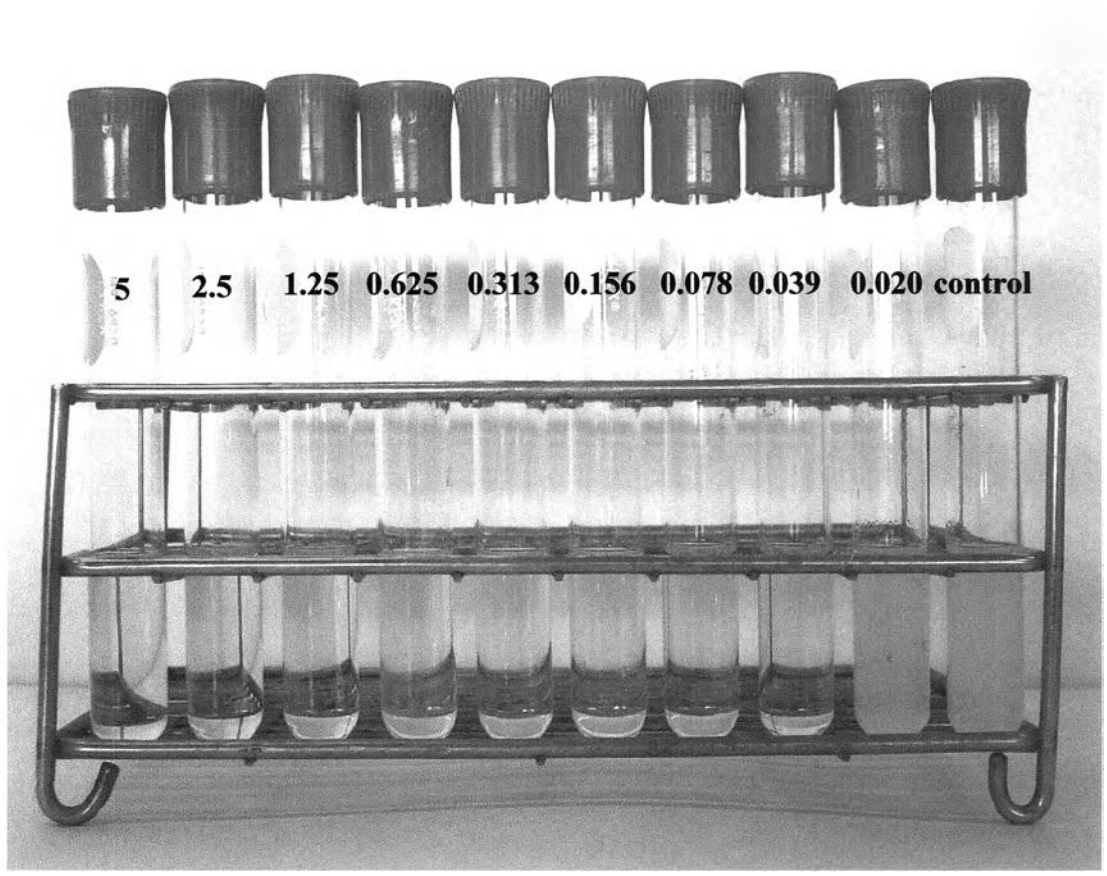


Figure 27. Broth macrodilution test for MIC value of betel vine oil against *S. epidermidis* ATCC 12228 no visible growth demonstrated at the lowest concentration 0.039% (v/v) of betel vine oil in MHB medium.

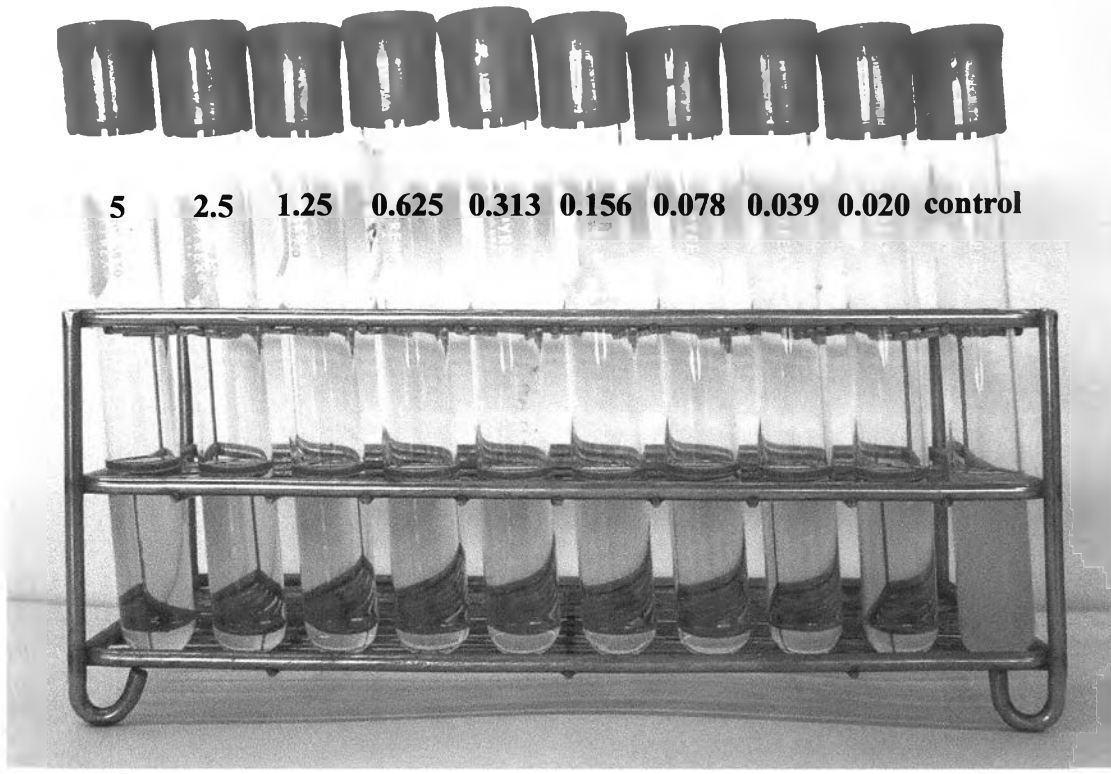


Figure 28. Broth macrodilution test for MIC of betel vine oil against *P. acnes*, no visible growth at the lowest concentration 0.020% (v/v) of betel vine oil in BHIB medium was demonstrated.

7. Surfactant optimization

The influence of the surfactant on the solution was studied using different kinds of surfactants such as Cremophor RH-40, Pluronic F-68 and Tween 80 at various concentrations (1, 5, 10 and 15% w/w). The solutions were examined by naked eye and the presence of aggregates or impurities was not detected. The transparent solutions were obtained using Cremophor RH-40, Pluronic F-68 and Tween 80 at concentrations 5, 10 and 15% w/w while 1% w/w of three surfactant gave cloudy solution. The results were shown in Table 11.

8. Formulation of antimicrobial PG preparation

Antimicrobial PG preparation was formulated by using two antimicrobial ingredients, polysaccharide gel (PG) and betel vine oil, in finish product. Since PG was water soluble but betel vine oil was oil soluble, then surfactant was required in the formulation. Different types of surfactant such as Cremophor RH-40, Pluronic F-68 and Tween 80 were tested. Types and concentrations of surfactant used and appearances of resulting products of PG with betel vine oil were indicated in appendix c.

In preparation of PG gel base and preparation of antimicrobial PG gel contained betel vine oil, PG used as gelling agent and also antimicrobial agent; Cremorphor RH-40, Pluronic F-68 and Tween 80 were used as surfactant in the preparation of antimicrobial PG preparations. Propylene glycol and glycerin were used as a humectant in water phase while amerchol L101 was used as an emollient in oil phase. In addition paraben concentrate was used as a preservative. Whereas triethanolamine was used to pH adjust. Betel vine oil acts as antimicrobial agent in the formulations. Antimicrobial PG preparation using Pluronic F-68 and Tween 80 was not form a stable preparation. In addition, tween 80 produced oily and tacky feeling after application, and Pluronic F-68 produced a greasy and sticky texture. Whereas, Cremorphor RH-40 produced a stable preparation. If more than 0.25% amerchol L101 was added in the formulations, it produced a glossy and non homogeneous texture. The formulations contained sorbitol was not stable and the

finished product was poorly spreaded. The formulations and description of finished products are demonstrated in Table 12.

Six preparations of PG gel contained organic acid were prepared using lactic acid and/or salicylic acid in various concentrations (0.1-1.0% w/v) are shown in Table 13-14. In the formulations that contained lactic acid and/or salicylic acid or their combination were successfully prepared, homogeneous preparations were obtained.

Preparations of PG gel contained solid antimicrobial agents such as precipitate sulphur and/or zinc oxide in various concentrations from 0.1 to 1.0% w/v were homogeneous but unstable. The preparation of PG gel contained antimicrobial agents and HPMC 4000 was also prepared by using precipitate sulphur and zinc oxide 0.5% and HPMC 4000 at concentrations 0.5 and 1.0%, the unstable finished products were obtained.

9. Physical properties evaluation of the finished products

The viscosity and pH values of the finished products were measured at ambient temperature and its appearances were recorded. The satisfactory products of antimicrobial PG preparation were indicated in Table 12-14 and appendix d. The pH of PG gel contained betel vine oil was not much different from PG gel base whereas the viscosities of the preparations were slightly increased. The pH values in preparation of PG gel contained organic acid, were slightly decreased, while their viscosities were increased.

Table 11. Compatibility test of betel vine oil with surfactant

Surfactants	Appearance
Tween 80 1%	Cloudy solution
Tween 80 5%	Transparent solution
Tween 80 10%	Transparent solution
Tween 80 15%	Transparent solution
Cremophor RH-40 1%	Cloudy solution
Cremophor RH-40 5%	Transparent solution
Cremophor RH-40 10%	Transparent solution
Cremophor RH-40 15%	Transparent solution
Pluronic F-68 1%	Cloudy solution
Pluronic F-68 5%	Transparent solution
Pluronic F-68 10%	Transparent solution
Pluronic F-68 15%	Transparent solution

Table 12. Formulation of PG gel base and formulation of PG gel base contained betel vine oil

Formulation No.	Ingredients (%w/w)								
	PG	Propylene glycol	Glycerin	Betel vine oil	Amerchol L101	Cremorphor RH-40	Triethanolamine	Paraben concentrate	Description of antimicrobial PG preparation After prepared
1 PG gel base	2.5	5	5	-	0.25	5	-	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 524 cps pH: 2.23
26 PG/betel 1%	2.5	5	5	1	0.25	5	-	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 600.8 cps pH: 2.21
45 PG/betel 2%	2.5	5	5	2	0.25	10	0.1	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 533.5 cps pH: 2.57

Table 13. Formulation of PG gel contained lactic acid

Formulation No.	Ingredients (%w/w)								
	PG	Propylene glycol	Glycerin	Betel vine oil	Amerchol L101	Cremorphor RH-40	Lactic acid	Paraben concentrate	Description of antimicrobial PG preparation After prepared
53 PG/lactic acid	2.5	5	5	1	0.25	5	0.4	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 660 cps pH: 2.12
54 PG/lactic acid	2.5	5	5	1	0.25	5	0.5	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 559.6 cps pH: 2.24
55 PG/lactic acid	2.5	5	5	1	0.25	5	1	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 490.6 cps pH: 2.16

Table 14. Formulation of PG gel contained salicylic acid

Formulation No.	Ingredients (%w/w)								
	PG	Propylene glycol	Glycerin	Betel vine oil	Amerchol L101	Cremorphor RH-40	Salicylic acid	Paraben concentrate	Description of antimicrobial PG preparation After prepared
59 PG/salicylic acid	2.5	5	5	1	0.25	5	0.4	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 490.8 cps pH: 2.23
60 PG/salicylic acid	2.5	5	5	1	0.25	5	0.5	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 457.2 cps pH: 2.26
61 PG/salicylic acid	2.5	5	5	1	0.25	5	1	1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 441.6 cps pH: 2.21

10. Assessment of antimicrobial PG preparation stability

All of formulations of antimicrobial PG preparations were prepared. The assessment of formulations was shown in Table 15 and appendix d. The antimicrobial PG preparations were stand at ambient temperature for 30 days. Not only that the antimicrobial PG preparations were vertically stored at 45 ^{o}C for 48 hours and then at -4 ^{o}C for 48 hours. This freeze-thaw cycle was continued for 6 cycles. The chosen formulation should neither be oily nor leave residue after application. The antimicrobial PG preparations using betel vine oil 1 and 2% w/v presented homogeneous aspect without aggregates, cloudy or phase separation. These formulae contained the same surfactant, which was Cremorphor RH-40, but different in the amount used. The stability under storage 30 days at ambient temperature and freeze-thaw cycles did not seem to affect pH. In contrast, the viscosity of the preparations were slightly increased (Fig 29A, 29B).

In preparation of PG gel contained organic acid, after testing at many conditions, the characteristics of gel were not changed. The physical appearance of preparation of PG gel contained organic acid showed a transparent gel. Both after 30 days stand at ambient temperature and after six freeze-thaw cycles still showed the same appearance as after freshly prepared. The pH values of PG preparations were slightly decreased, while their viscosities were increased after assessment of antimicrobial PG preparation stability. The preparations of PG gel with lactic acid and/or salicylic acid were illustrated in Fig 30A, 30B.

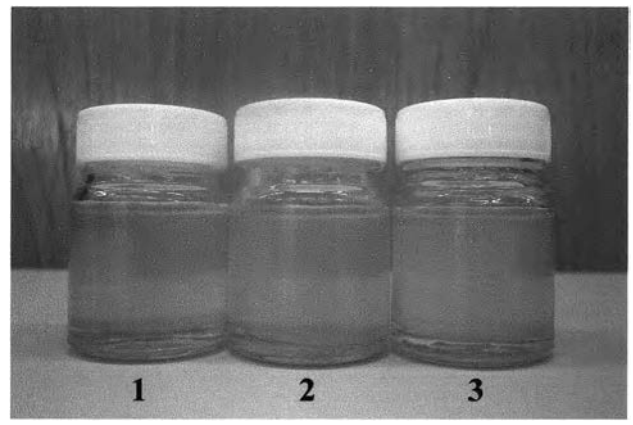
For preparation of PG gel contained antimicrobial agents (insoluble), the physical appearance after prepared of six formulae with precipitate sulphur shown yellow suspension with high viscosity. Whereas formulations with zinc oxide were white suspension with high viscosity. Formulations with precipitate sulphur and/or zinc oxide could not form preparations as the separations of water phase, oil phase and aggregates occurred. The stability under storage at ambient temperature for 30 days and after six freeze-thaw cycles slightly decreased pH of formulations with precipitate sulphur but more increased pH in the formulations with zinc oxide whereas the viscosities were increased.

Table 15. Assessment of antimicrobial PG preparation stability

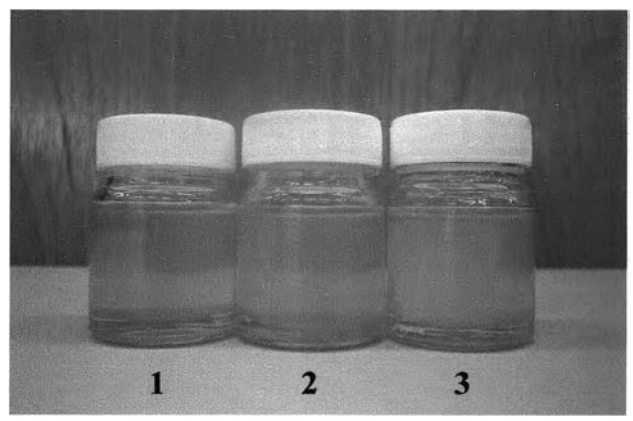
Formulation No.	Description of formulation		
	Freshly prepared	After 30 days stand at ambient temperature	After 6 freeze-thaw cycles
1	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 524 cps pH: 2.23	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 4592 cps pH: 2.17	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 4862 cps pH: 2.17
26	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 600.8 cps pH: 2.21	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 4075 cps pH: 2.28	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 4720 cps pH: 2.20
45	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 533.5 cps pH: 2.57	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 6193 cps pH: 2.12	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 7024 cps pH: 2.15
53	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 660 cps pH: 2.12	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 5620 cps pH: 2.11	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 6950 cps pH: 2.13
54	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 559.6 cps pH: 2.14	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 5012 cps pH: 2.16	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 6856 cps pH: 2.15
55	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 490.6 cps pH: 2.16	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 5140 cps pH: 2.16	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 5985 cps pH: 2.15

Table 15. Assessment of antimicrobial PG preparation stability (continued)

Formulation No.	Description of formulation		
	Freshly prepared	After 30 days stand at ambient temperature	After 6 freeze-thaw cycles
59	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 490.8 cps pH: 2.23	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 7337 cps pH: 2.27	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 8980 cps pH: 2.27
60	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 457.2 cps pH: 2.26	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 7457 cps pH: 2.27	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 8879 cps pH: 2.28
61	Appearance: homogenous Flow: easy Air bubbles: non Color: pale brown Viscosity: 441.6 cps pH: 2.21	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 7318 cps pH: 2.27	Appearance: homogenous Flow: not easy Air bubbles: non Color: pale brown Viscosity: 8092 cps pH: 2.28

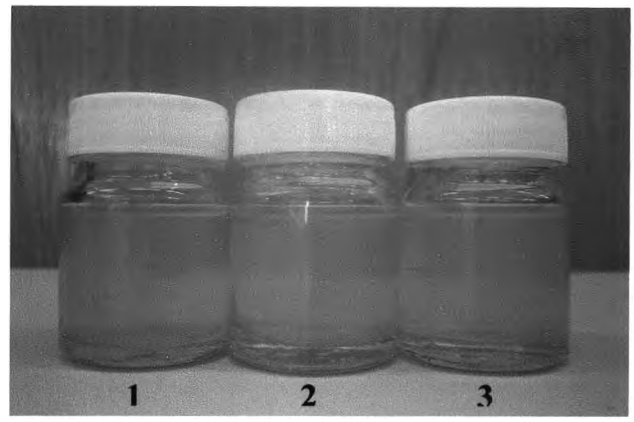


(A)

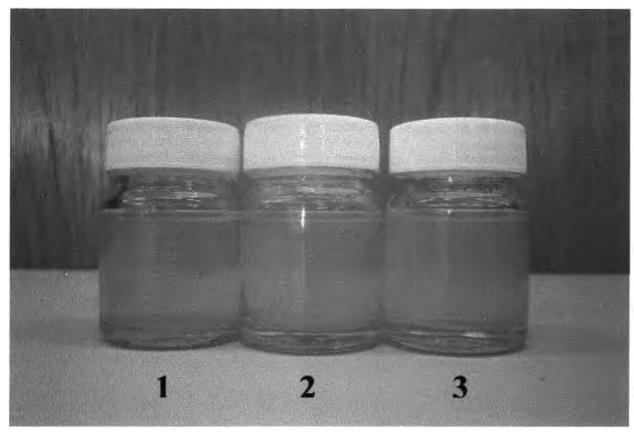


(B)

Figure 29. Antimicrobial PG preparation: (A) contained betel vine oil 1%, (B) contained betel vine oil 2%; 1=Freshly prepared, 2=After stand 30 days, 3=After six freeze-thaw cycles



(A)



(B)

Figure 30. Antimicrobial PG preparation: (A) contained lactic acid 0.5%, (B) contained salicylic acid 0.5%; 1=Freshly prepared, 2= After stand 30 days, 3= After six freeze-thaw cycles

11. Bacterial susceptibility tests of the finished products antimicrobial PG preparation

11.1 Agar well diffusion method

The inhibition zones from different concentration (1 and 2%) of betel vine oil in antimicrobial PG preparation were determined, PG base preparation was a control. Betel vine oil at 1 and 2% concentration of 2.5% PG and Panoxyl 5® gel were used as standard. The results are shown in Table 16 and Figure 31. Standard anti-acne gel (Panoxyl 5® gel) was simultaneously used as positive control. The inhibition zones of antimicrobial PG preparation contained 1% betel vine oil were 10.10 (0.10), 10.35 (0.13) and 10.70 (0.15) mm against *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis*, respectively. Additionally, the inhibition zones of antimicrobial PG preparation contained 2% betel vine oil against *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis* were 10.52 (0.08), 10.58 (0.08) and 10.95 (0.05) mm, respectively. The results suggested that comparing the various sample investigated, at 2% concentration of betel vine oil exhibited a comparable antibacterial activity PG with 2% betel vine oil and positive control of Panoxyl 5® gel. The antimicrobial PG preparation contained 1% betel vine oil and 2.5% PG solution also exhibited inhibition zones against these microorganisms. Whereas the negative control PG preparation base exhibited no inhibition zone.

Table 16. Antibacterial activity of various samples on growth of bacteria by agar well diffusion method

Samples	Diameter of inhibition zone, mm mean (SD)		
	P. acnes	*S. aureus*	*S. epidermidis*
No. 26 antimicrobial PG preparation with 1% betel vine oil	10.10 (0.10)	10.35 (0.13)	10.70 (0.15)
No. 45 antimicrobial PG preparation with 2% betel vine oil	10.52 (0.08)	10.58 (0.08)	10.95 (0.05)
No. 1 PG base preparation	NZ	NZ	NZ
1% betel vine oil in 0.1% tween 80	10.55 (0.13)	10.90 (0.09)	10.92 (0.06)
2% betel vine oil in 0.1% tween 80	11.48 (0.16)	11.73 (0.10)	11.72 (0.08)
2.5% PG	10.20 (0.09)	10.52 (0.23)	10.40 (0.10)
Panoxyl 5® gel	15.18 (0.14)	15.28 (0.18)	20.03 (0.06)

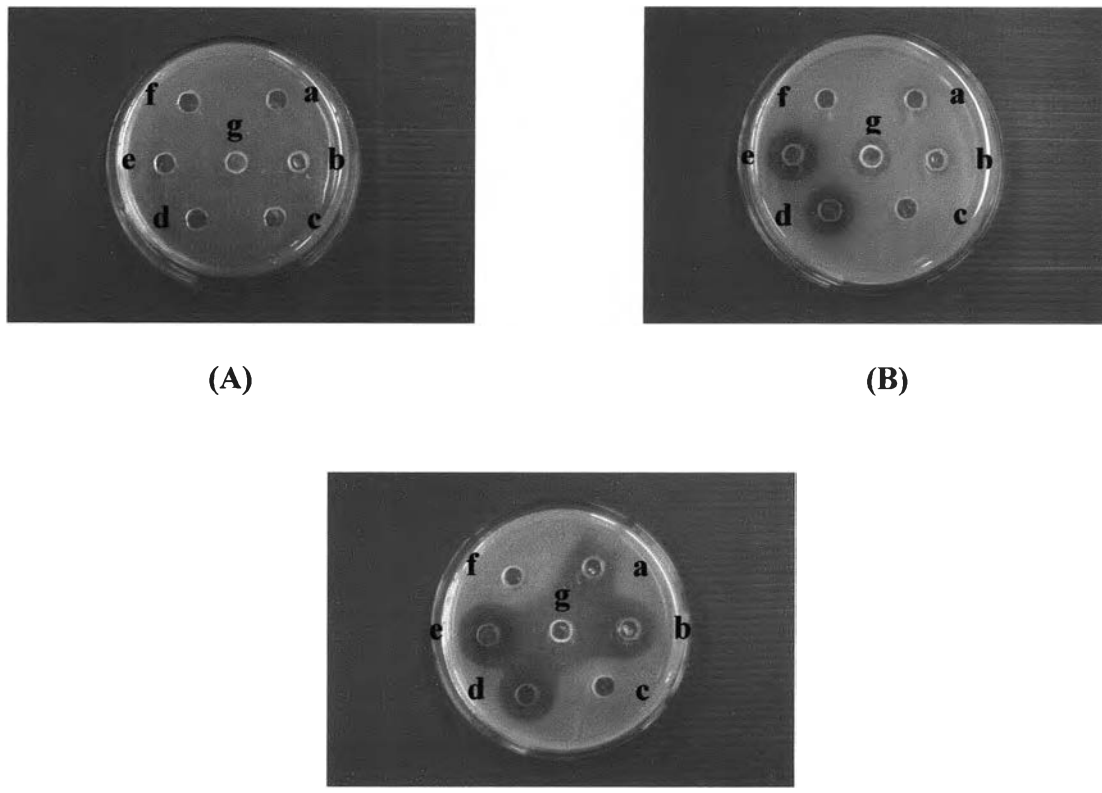


Figure 31. Microbiological assay plate of various samples against (A) *Propionibacterium acnes*, (B) *Staphylococcus aureus*, (C) *Staphylococcus epidermidis*; a- antimicrobial PG preparation contained 1% betel vine oil (No. 26), b- antimicrobial PG preparation contained 2% betel vine oil (No. 45), c- PG base preparation (No. 1), d-1% betel vine oil, e-2% betel vine oil, f- 2.5% PG, g- Panoxyl 5® gel

11.2 Broth microdilution assay

The MIC and MBC values of different products were also determined against *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis*. The results are shown in Table 17 and Figure 32. The MIC and MBC values of antimicrobial PG preparation contained 1% betel vine oil were noted against *Propionibacterium acnes* and *Staphylococcus aureus* were at 20 and 100% of finished products (No. 26), against *Staphylococcus epidermidis* were at 4 and 20% betel vine oil of finished products (No. 26). In addition, MIC and MBC values against *Propionibacterium acnes* and *Staphylococcus epidermidis* were at 4 and 20% of finished products antimicrobial PG preparation contained 2% betel vine oil (No. 45) against. While MIC and MBC value were at 20 and 100% of product antimicrobial PG preparation contained 2% betel vine oil (No. 45). The 2.5% PG showed MIC and MBC values against *Propionibacterium acnes, Staphylococcus aureus* and *Staphylococcus epidermidis* at 20 and 100% of 2.5% PG dispersion (equivalent to 0.5 and 2.5% PG concentration), respectively. PG preparation base of full strength was not exhibit visible inhibition. The positive control (Panoxyl 5® gel) exhibited the strongest antibacterial activity according to this study.

Table 17. Minimal inhibitory concentrations (MIC) and Minimal bactericidal concentrations (MBC) of finished products and ingredients against microorganisms by broth microdilution method

Sample	MIC (% finished products)			MBC (% finished products)		
	P. acnes	*S. aureus*	*S. epidermidis*	*P. acnes*	*S. aureus*	*S. epidermidis*
No. 26 antimicrobial PG preparation contained 1% betel vine oil	20	20	4	100	100	20
No. 45 antimicrobial PG preparation contained 2% betel vine oil	4	20	4	20	100	20
No. 1 PG base preparation	-	-	-	-	-	-
1% betel vine oil in 0.1% tween 80	4	20	0.8	20	100	4
2% betel vine oil in 0.1% tween 80	0.8	4	0.8	4	20	4
2.5% PG	20	20	20	100	100	100
Panoxyl 5® gel (positive control)	0.16	0.8	0.16	0.8	4	0.8

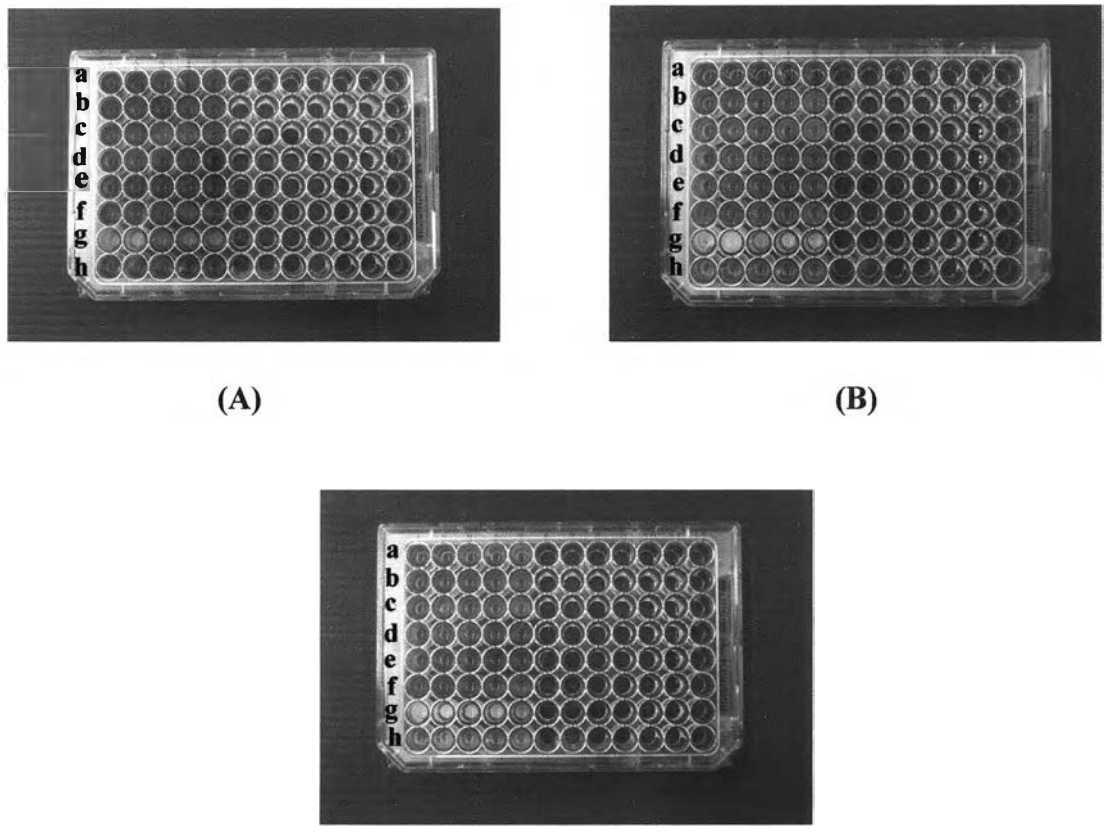


Figure 32. Broth microdilution test for MIC of various sample against (A) *Propionibacterium acnes*, (B) *Staphylococcus aureus*, (C) *Staphylococcus epidermidis*; a- antimicrobial PG preparation contained 1% betel vine oil (No. 26), b- antimicrobial PG preparation contained 2% betel vine oil (No. 45), c-PG base preparation (No. 1), d- 1% betel vine oil, e- 2% betel vine oil, f- 2.5% PG, g- Panoxyl 5® gel, h-control