CHAPTER IV

DISCUSSION AND CONCLUSION

Discussion

1. Isolation of Polysaccharide Gel (PG)

The final yield of semi-purified polysaccharide gel (PG) was 6.72% by weight of dried fruit-rinds. The powder of polysaccharide gel was passed through 60 meshes. The pale brown powder was obtained. The yield and appearance of semi-purified PG obtained was about the same as previous studied (Gerddit, 2002). The polysaccharide gel have found to compose of long chain of polygalacturonic acid branches with neutral sugar such as glucose fructose rhamnose and xylose (Hokputsa *et.al.*, 2004). The polysaccharide have found soluble in water and formed a viscous gel of high molecular weight 400-1400 kDa macromolecule (Gerddit, 2002). Antibacterial activity of the polysaccharide gel has been explored (Lipipun *et al.*, 2002; Pongsamart *et al.*, 2005). This biomaterial is expected to be useful for varieties of pharmaceuticals of antibacterial purpose.

2. Properties of Polysaccharide gel (PG)

2.1 Viscosity

The polysaccharide gel (PG) dispersion at concentration 2.5% by weight in water exhibited 528 ± 2.00 cps viscosity. According to the previous study (Lertchaiporn, 2003), PG concentration increase resulted in viscosity increase this behavior of PG was found similarly to other polysaccharide derivatives such as methylcellulose (MC), carboxymethylcellulose (CMC) and hydroxypropyl methylcellulose (HPMC). The materials belong to this property have been widely used as thickening, gelling and suspending agent in cosmetic industries (Chaplin, 2004). PG of durian fruit-rind has also been expected to be used for cosmetics and pharmaceuticals purpose.

2.2 pH

PG at 2.5% by weight in water showed pH 2.10 ± 0.03, whereas pH of PG at low concentration was about 2.20-2.30. The result was agreed with the previous studied (Lertchaiporn, 2003). This result suggested the influences of major acidic sugar, galacturonic acid, in the polysaccharide gel (PG) of durian fruit-rind. Due to the dominant feature of polysaccharide gel consist mainly of a linear chain of (1 → 4) glycosidic linked galacturonic acid units (Hokputsa *et.al.*, 2004). The PG products contained considerable amount (68%) of acidic chain of polygalacturonan.

2.3 Effect of sorbitol on viscosity of PG

The effect of sorbitol on the viscosity of PG was evaluated. The viscosity of PG was increased with respected to the increasing of sorbitol concentration. The concentration of sorbitol using in the formulation was not much effected to the viscosity of PG. The sorbitol was used as humectants and a solubilizing as well as propylene glycol. Addition of humectants in preparation of hand cleansing gel helped to prevent skin irritation that can discourage hand washing (Brannan, 1997). In this study, the antiseptic PG gel preparation used only propylene glycol due to addition of sorbitol in the formulations produced an unstable and sticky product.

3. Properties of tea tree oil (TTO)

3.1 Compatibility with solubilizer

In order to test antimicrobial activity of water insoluble materials such as essential oil, it is necessary to incorporate an emulsifier or organic solvent to dissolve tested materials into the test medium and to ensure contact between the test organism and the tested materials in the experiment designed. In this study, the stability of the emulsions was determined by mixing with various type of solubilizer such as, 0.5% Tween 20, 10% Tween 20, 0.5% Tween 60, 10% Tween 60, 0.5% Tween 80, 10% Tween 80, 10% Cremophore RH40® and 15%Cremophore RH40®. The results indicated that the concentration of 0.5% Tween 80 and 10%-15% Cremophore RH40® were necessarily to maintain stability of 1 % tea tree oil (TTO) with in emulsion.

Lipophilic molecule including in the components of tea tree oil may be formed layer within the micelles by non-ionic surfactant, such as Tween 80 and Tween 20, and are thus partitioned out of the aqueous phase of the emulsion (Schmolka, 1973). The more marked effect is produced at higher concentrations of surfactant (Van Doome, 1990).

3.2 Antimicrobial activity test

The *in vitro* antimicrobial activity of tea tree oil (TTO) was determined against 9 bacteria and 2 yeast strains. Four strains of gram-positive bacteria, *S. aureus* and *S. epidermidis* are the cause of skin infection and pus; *M. luteus* and *B. subtilis* can be found in skin as normal flora in environment. Five strains of gram-negative bacteria, *E.coli* and *P. vulgaris* can be found in gastrointestinal tract as normal flora; *S.typhimurium* is the cause of food poisoning; *K. pneumoniae* and *Ps. aeruginosa* can cause infection in immunocompromised individuals. Two yeast strains, *S. cerevisiae* can be found in the environment, whereas *C. albican*s can cause skin and oral infection in healthy individuals. Tea tree oil showed inhibitory activity against 9 bacteria strains and 2 fungi by agar diffusion and broth macrodilution tests. Antimicrobial activity of TTO provides a good thought to develop the formula of valuable antiseptic products in combination with PG.

3.2.1 Agar diffusion test

Inhibition zone was observed on agar plates with tea tree oil at concentration 0.312- 0.625% (v/v) against *S. aureus*, *S. epidermidis*, *M. luteus*, *B. subtilis*, *E. coli*, *S. typhimurium*, *P. vulgaris*, *K. pneumoniae*, *Ps. aeruginosa*, *S. cerevisiae* and *C. albicans*. An increment of inhibition zone diameter was found with respected to increasing concentration of tea tree oil. Inhibitory activity of tea tree oil against gram-positive and gram-negative bacteria and fungi was determined. The results were corresponded to the previous studied by Carson *et al.*, (1995). Inhibitory effect of tea tree oil against fungi was as effective as bacteria the inhibition zones of the test fungi strain were larger at higher concentrations. In this study, the widest inhibition zone of tea tree oil was obtained against *C. albicans* (diameter = 15.94 ±

0.12 mm.) and the smallest inhibition zones of tea tree oil were obtained against *E .coli* (diameter = 10.33± 0.02 mm.). Tea tree oil is a complex mixture of hydrocarbons and terpenes consisting of approximately 100 components. There are eight major components, which terpinen-4-ol is regarded as the main major antimicrobial component (Carson, Riely, 1995). However, Carson *et al.*, (1995) suggested that 1, 8-cineole may be enhance antifungal activity. In order to maintain contact between the oil and the microorganisms throughout the tested period, the stability of oil in the emulsion was necessary. This study, 0.5% Tween 80 was added in tea tree oil to enhance oil solubility and 0.5% Tween 80 was used as control, which Tween 80 was not showed inhibitory activity against all microorganisms.

3.2.2 Broth macrodilution test

Tea tree oil provides broad-spectrum antimicrobial activity. The results of MIC and MBC values were correlated with the previous studied by Carson *et al.*, (1995). MICs and MBCs of tea tree oil against nine bacteria and two fungi were determined. MICs of tea tree oil were at 0.036-0.3125% (v/v) and MBCs of tea tree oil were at 0.078-0.625% (v/v) against the tested microorganisms. *S. typhimurium* showed the most susceptible to tea tree oil with the values of MICs and MBCs at 0.036% and 0.078% (v/v), respectively. *M. luteus*, *B. subtilis*, *P. vulgaris*, *K. pneumoniae* and *S. cerevisiae* were low susceptible to tea tree oil with the values of MICs and MBCs at 0.3125% and 0.625%, respectively. The present data of MIC and MBC values were similar to those reported in the previous studied. The result indicated that variety of microorganisms were susceptible to tea tree oil (Carson, Riley, 1993). The results were correlated with the previous studied by Hammer *et al.*, (1996). In this study, *S. typhimurium* was the most susceptible to tea tree oil compared with other microorganisms. This result was similar to the previous study of Carson, Riely, (1995). According to other studied, they have suggested that tea tree oil might be inhibiting growth of bacteria and fungi by damage to the cell membrane or cell wall, which causes leakage of potassium ions (Riley, 1998).

The MIC and MBC values obtained in this study did not present the same values according to those of other studies due to the conditions were

different such as the test method, the medium used in assay, and the criteria or method used for determining the inhibitory activity.

4. Preparation of antiseptic PG gel

Antiseptic PG gel preparations were formulated by using antibacterial polysaccharide gel (PG) from durian fruit-rind together tea tree oil and betel oil as an additional active antimicrobial agent and the other ingredient such as propylene glycol as a humectants, vitamin E as an antioxidant, Amerchol L-101 as an emollient, paraben concentrate as a preservative, menthol as a cool sensation and Cremophore RH-40® as a sulubilizer. The antiseptic PG gel products contained 2.5% PG, 0.05 M. $CaCl_2$ and an appropriate amount of triethanolamine (TEA) to make pH 2.9 ± 0.1. The antiseptic PG gel products NO.12, 33 and 43 were satisfactory prepared and selected (Table 8). The texture of gel finished products were homogenously smooth, natural colored, easily flowed, non-sticky and provided antimicrobial activity. Adding of sorbitol and glycerin in formula made the non-stable gel product and non-homogenous gel formation. Whereas Tween 80 produced the sticky gel product. Addition of ethanol in the formula made the unstable gel product and precipitated. The selection of the antiseptic gel final product needed to consider a good appearance, stability, antimicrobial activity and applicability of the product to the skin.

5. Stability test of antiseptic PG gel products

The stability test of antiseptic PG gel products were determined. The first method was performed by freeze-thaw method, the products were kept at 45°C for 48 hrs and transfer to keep in low temperature at -4°C for 48 hrs to complete 1 cycle. The procedure was performed for 6 cycles. The second method was tested by storage at ambient temperature for 30 days. The gel viscosity and pH of resulting products were measured. The formulation NO.12, 33 and 43 were selected after stability test. The physical properties of antiseptic gel products were not unacceptable changed. The viscosity and pH values of all formulations (NO.12, 33 and 43) were determined after storage, however, longer time need to be observed. The viscosity and pH values of the products (NO.12, 33 and 43) after storage were higher than freshly prepared

products which was due to the rheological property of PG as a non-newtonian of pseudoplastic behavior (Lertchaiporn, 2003). The freshly prepared product of other formulations in appendix C were precipitated, non-homogenous and unstable while the formulation NO.12, 33 and 43 were acceptable.

6. Efficacy of antiseptic PG gel

6.1 Time-kill analysis

Bactericidal activity of the final products of antiseptic PG gel was determined against 2 bacteria strains, *S. aureus* that represent gram-positive, *E. coli* that represent gram-negative bacteria and *C. albicans* a representative of fungi. Time-kill analysis is an extension of the MBCs and gives information about the rates at which organisms are killed. Time-kill analysis illustrated that antiseptic gel preparation of tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43) gave bactericidal activity in medium MHB against *S. aureus* and *E. coli*, the colony counts were declined to zero within 15 min. Whereas NSS exhibited that all tested microorganisms were viable according to the survival pattern in time-kill assay. This result was agreeable to the results of other laboratories (Carson, Riley 1995). The final products of antiseptic PG gel showed more effective antimicrobial activity than PG gel base (without menthol).

Antiseptic gel preparations of tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43) also possessed fungicidal activity in SDA medium against *C.albicans*, the colony counts were declined to zero within 6-24 hrs. The time-kill assay indicated that fungi were not as rapidly killed as bacteria, even at several times higher concentration of its minimal fungicidal concentration (MFC) value, the time of exposure may play a significant role for fungicidal activity of antiseptic PG gel products. Broader antimicrobial activity of antiseptic PG gel product might suggest that the enhancing of the combination of the three antimicrobial agents used in the formula.

6.2 Agar diffusion method

Antimicrobial activity of tea tree oil (TTO) antiseptic gel final products were studied against 9 bacteria strains, *Staphylococcus aureus* ATCC 6538P, *Staphylococcus epidermidis* ATCC 12228, *Micrococus luteus* ATCC 9341, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC 25922, *Salmonella typhimurium* ATCC 14028, *Proteus vulgaris* ATCC 13315, *Klebsiella pneumoniae* ATCC 10031 and *Pseudomonas aeruginosa* ATCC 9721 and two yeast strains, *Saccharomyces cerevisiae* ATCC 9763 and *Candida albicans* ATCC 10230, respectively. The inhibition zones were determined on the agar plates, NSS was control, PG-gel base contained menthol (NO.12), 1% tea tree oil-PG gel (NO.33), 1.5% tea tree oil-PG gel (NO.35) and tea tree oil/betel oil-PG gel (NO.43) were tested. No inhibition zone of tested microorganisms was observed with the control NSS. The results showed that, tea tree oil at higher concentration demonstrated larger inhibition zone. In comparison of inhibition zones between 1% tea tree oil-PG gel (NO.33) and 1.5% tea tree oil-PG gel product (NO.35) demonstrated that, 1.5% tea tree oil-PG gel product (NO.35) showed wider inhibition zone than the 1% tea tree oil-PG gel product (NO.33). Whereas, comparison of inhibition zones between 1% tea tree oil-PG gel (NO.33) and Tea tree oil/betel oil-PG gel product (NO.43) demonstrated that, Tea tree oil/betel oil-PG gel product (NO.43) showed wider inhibition zone than the 1% tea tree oil-PG gel product (NO.33). Tea tree oil/betel oil-PG gel product (NO.43) which was 1%TTO and 0.2% of betel oil also provided as good inhibitory activity due to the combined antimicrobial activity of betel oil (Remington *et al.*, 1918) as well as tea tree oil.

All ingredients in the formula were also tested for microorganisms-free and inhibitory activity against test microorganisms, such as *S. aureus*, *S. epidermidis*, *M. luteus*, *B. subtilis*, *S. typhimurium*, *P. vulgaris*, *K. pneumoniae* and *Ps. aeruginosa*. The results demonstrated that all ingredients were free of microorganisms and no inhibitory activity against test microorganisms, except for that of menthol. Menthol showed inhibitory activity against most of microorganisms, except *E. coli*, *C. albilcans* and *S. cerevisiae*. Menthol has been found possess antibacterial activity

(Karapinar and Aktug, 1987; Moleyar and Narasimham, 1992). The present of 1.2% menthol in the formulas (NO12, 33, 35 and 43) probably enhanced the antimicrobial activity of antiseptic TTO-PG gel products.

6.3 In-vivo hand washing test

In-vivo test for bacterial inhibition was evaluated by the colony counts of the any microorganisms released from tested hands was which fingertip contaminated on agar plates before and after application of antiseptic PG gel products (NO.33 and NO.43) and PG-gel base (NO.12). The prevalue is the number of colony forming unit (CFU) sampling from the fingertips before treatment, and the postvalue is the number after treatment with antiseptic PG gel products. Antiseptic activity of antiseptic PG gel contained tea tree oil/betel oil (NO.43), antiseptic PG gel contained tea tree oil (NO.33) and PG-gel base (NO.12) were evaluated by the reduction of number of colony counts. The results showed less count after washing hands with product NO.43 and 33. The number of released microorganisms was also depended on condition and time tested, final products, steps of hand washing, environmental contamination of microorganisms from air and water, types of normal flora and number of transient skin flora. The previous works have suggested that the repeated use of TTO-contained products have not lead to the dermatological problems associated with other formulations (Carson, Riley, 1996). Hence, it was anticipated that the hand antiseptic PG gel products in this study have potential to be useful to decontaminate opportunity microorganisms while preserving the naturally occurring flora.

Antiseptic activity of antiseptic PG gel contained tea tree oil/betel oil (NO.43), antiseptic PG gel contained tea tree oil (NO.33) were also determined in comparison with gel commercial product by hand washing test, tap water was control. The results showed that less colony count were observed after washing hands with product NO.43, 33 and gel commercial. In-vivo results showed that antiseptic gel product (NO.33 and 43) and gel commercial were similarly effective to reduce hands normal flora but more effective than tap water. The results showed that antiseptic PG gel

products have antibacterial activity comparable with gel commercial used in this study.

6.4 Perception analysis of antiseptic PG gel products

The perception analysis of antiseptic PG gel product (NO.33 and 43) in volunteers revealed that most of them satisfied with the antiseptic PG gel NO. 33 and NO.43. The antiseptic PG gel NO.33, the first and second ranks of satisfaction was smoothness and clearness, respectively. And antiseptic PG gel NO.43, the first and second ranks of satisfaction was firmness and smoothness, respectively. After finishing the test, the volunteers were also satisfied with the physical appearance of both antiseptic PG gel (NO.33 and 43). Furthermore, they were interested in using antiseptic PG gel due to its natural origin.

Conclusion

Polysaccharide gel (PG) isolated from fruit-rinds of durian (*Durio zibethenus* Murr.) (Smittinand, 2001) is a natural heteropolysaccharide composes of sugars including galacturonic acid in polygalacturonan chain, with branches of neutral sugars such as arabinose, rhamnose, fructose and glucose (Gerddit, 2002; Hokputsa *et al.*, 2004). The antibacterial activity of polysaccharide gel (PG) from fruit-rinds of durian against microorganisms has been reported. PG at 2.5% in water showed bactericidal effect against bacteria such as *S. aureus*, *B. subtilis*, *M. luteus*, *S. epidermidis*, *E.coli*, *P. vulgaris* and *L. pentosus* (Nantawanit, 2001). The PG powder is light-brown in color, formed a viscous gel in water and acidic pH. The viscosity of PG increased with respect to the increasing of sorbitol concentration. The concentration of sorbitol used in the formulation was not much changed to the viscosity of PG.

PG was found compatible with solubilizer, 10%-15%Cremophore RH40, clear solution, which was used in the formulation of antiseptic PG gel products.

The antimicrobial activity of tea tree oil (*Melaleuca alternifolia* Cheel) by agar diffusion susceptibility test against *S. aureus*, *Ps. aeruginosa*, *S. epidermidis*, *M.*

luteus, *E. coli*, *B. subtilis*, *P. vulgaris*, *S. typhimurium*, *K. pneumoniae* and two yeast strains, *Candida albicans* and *Saccharomyces cerevisiae*, demonstrated that tea tree oil at 0.312% (v/v) demonstrated provided clear inhibition zone on agar plates. Broth macrodilution method was performed to determine minimal inhibitory concentration (MIC) and minimal bactericidal concentration (MBC) of tea tree oil was against microorganisms in a range of 0.036% to 0.312% and 0.078 % to 0.625%, respectively.

Antiseptic PG gel preparations was formulated by using 2.5% PG as a gelling and antibacterial agent, tea tree oil and betel oil as an active antimicrobial agent, the other ingredients such as propylene glycol was used as a humectants, vitamin E was an antioxidant, Amerchol L-101 was an emollient, menthol was for a cool sensation and Cremophore RH-40 was a sulubilizer. Viscosity and pH of finished products were adjusted by using an appropriate amount of 0.05 M $CaCl_2$ and triethanolamine (TEA), respectively.

The formulation NO.12, 33 and 43 were successfully prepared, good appearance and stable products were obtained. The texture of gel finished products were homogenously smooth, pale brown colored, easily flowed, non-sticky and provided antimicrobial activity. The stability test of antiseptic PG gel after 6 Freeze-thaw temperature cycles (-4°C, 24 hr; 45°C, 24hr) or storage at ambient temperature for 30 days demonstrate the stable products

Antiseptic gel preparation was formulated and successfully prepared by using the combination of PG, tea tree oil and betel oil as the active antimicrobial agents. Bactericidal activity of the of antiseptic PG gel products was demonstrated by time-kill analysis, *S. aureus* and *E. coli*, were completely killed by the preparation of tea tree oil-PG gel (NO.33) and tea tree oil/betel oil-PG gel (NO.43) within 15 min. Whereas the colony counts of *C. albicans*, was declined to zero with tea tree oil-PG gel and tea tree oil/betel oil-PG gel products within 6-24 hours.

The products of antiseptic gel including PG-gel base contained menthol (NO.12), 1% tea tree oil-PG gel (NO.33), 1.5% tea tree oil-PG gel (NO.35) and tea

tree oil/betel oil-PG gel (NO.43) exhibited growth inhibition against all tested bacteria and fungi, inhibition zones on agar plates were demonstrated.

In addition, menthol which was added for cool sensation and essence purpose also showed inhibitory activity against most of tested microorganisms, except for *E. coli*, *C. albicans* and *S. cerevisiae.*

In-vivo study of antiseptic potency of antiseptic PG gel products (NO.33 and NO.43) and PG-gel base (NO.12) indicated that skin normal flora on hands reduced after washing hands with products NO.43 and 33 more than washing hand with tap water.

Antiseptic efficacy of antiseptic PG gel product was evaluated in comparison with gel commercial product in 10 subjects. The results showed that less colony count were observed after washing hands with product NO.43, 33 and gel commercial. In-vivo results showed that antiseptic gel product (NO.33 and 43) and gel commercial were similarly effective to reduce hands normal flora but more effective than tap water. The results showed that antiseptic PG gel products have antibacterial activity comparable with gel commercial used in this study.

The perception analysis of antiseptic PG gel product (NO.33 and 43) by volunteers revealed that most of them were satisfied with the antiseptic PG gel NO. 33 and NO.43. After finishing the test, the volunteers were also satisfied with the physical appearance of both antiseptic PG gel (NO.33 and 43). Furthermore, they were interested in using antiseptic PG gel due to its natural origin.