CHAPTER IV

DISCUSSIONS AND CONCLUSIONS

DISCUSSIONS

The yields of polysaccharide gel (PG) extracted from fruit-rinds of Durian (*Durio zibethinus* Murr.) were approximately 2% w/w from fresh rinds. PG is a heteropolysaccharide composes of complex mixture of glucose (20.9%), rhamnose (4.8%), galactose (4.9%), xylose (0.4%), rhamnose (4.8%), arabinose (1.2%) and galacturonic acid (67.9%) (Pongsamart and Panmaung, 1998; Girddit et al, 2001). Galacturonic acid is major components, with is regarded as the main major antibacterial component. However, resulting product of PG made of natural material seemed to provide an alternative agent for treatment of bacterial infection for cow mastitis.

Totally five bacterial strains, *Staphylococcus* spp., *Streptococcus* spp., *E. coli, K. pneumoniae* and *Pseudomonas* spp. was used in this investigation. According to our knowledge, there was a few previous literature on antibacterial properties of polysaccharide of durian extracts. Xiaohui W. *et. al.,* 2004 have assay antibacterial activity of chitin-chitosan, against *S. aureus* and *E. coli*. According to another report the extract of active polysaccharide from aloe vera showed antibacterial activity against *S. aureus* and *E. coli* (T. Reynolds and A. C. Dweck, 1999). These results confirmed the result of earlier studies that some natural polysaccharide extracts had antibacterial activity.

1. Agar diffusion

The results of susceptibility test of *Staphylococcus* spp. and *Streptococcus* spp. to PG demonstrated that at higher concentration of PG provided bigger inhibition zone followed the sequence; 5>2.5>1.25>0.625>0.32% PG, while *E. coli* was less susceptible to PG. PG at high concentrations affected the growth of all of the organisms tested.

An increasing of inhibition zone diameter was obtained with respect to the increasing concentrations of PG. Several factors that effect the diffusion capacity of ingredients in agar plates must be considered, such as the contact between experimental material and agar, molecular weight, size and shape of the antibacterial agent, load and concentration of test material, agar gel viscosity and ionic concentration in relation to medium. Furthermore, control and standardization of inoculation density, evaluation of results, selection of agar medium, selection of microorganisms, dept of agar medium, incubation temperature of plate and reading point of inhibition zone are also restricting factors affecting the dynamic and variability of diffusion tests in an agar medium (Leonardo *et al.*, 2000).

The *in vitro* antibacterial activity of PG against the bacteria, employed and its activity potentials were qualitativelty and quantitatively assessed by the presence or absence of inhibition zone, zone diameters and MIC values. According to the results given in Table 8, 10, 12, 14 and 16 PG against mastitis cow isolates was demonstrated by susceptibility test, *in vitro* antibacterial activities of PG was observed against all five bacteria genus tested. The data obtained from the agar diffusion method indicated that the PG displayed a variable degree of antibacterial activity on different tested strains. The inhibitory effect increased with the increasing of the PG concentration from 25-50 mg/ml. The data indicated that gram positive bacteria (*Staphylococcus* spp. and *Streptococcus* spp.) were more sensitive tested strain than gram negative tested strain to the PG of durian rinds, represented by wider diameter of inhibition zones. The main antibacterial constituent of PG has been considered due to acidic effect of the galacturonic acid component in PG, which is usually referred to as acidic polysaccharide.

2. MIC/MBC of PG Monthong

The antibacterial activity of PG against bacteria isolated from cow mastitis was investigated by determining the MIC (minimum inhibitory concentration) and MBC (minimum bactericidal concentration) values of PG using the broth microdilution method. Values represented MICs of PG incomparison with galacturonic acid against *Staphylococcus* spp., *Streptococcus* spp., *Pseudomonas* spp., *E.coli* and *Klebsiella* spp. are demonstrated in Table 8, 10, 12, 14 and 16 gentamicin was a positive control. The

results showed that all strains of bacterial isolates from cow mastitis showed a comparable susceptibility to PG. The average MBC values of PG against most of the tested bacteria was 12.5-6.25 mg/ml. MBC values of PG were mostly 2 times higher concentration than its MIC values. The results were clearly demonstrated that most of the *S. aureus* isolates were more susceptible to PG than laboratory tested strains of *S. aureus* ATCC 25923 according the previous studies (Lipipun, *et. al.*, 2002 and Pongsamart *et. al.*, 2005). PG showed a comparable activity against tested gram positive and gram negative bacteria. In addition, the isolated of Streptococci, *Pseudomonas* spp. and *K. pneumoniae* that exhibited resistance to gentamicin (MBC ≥8 µg/ml, Lorian 1991) were found susceptible to PG with the MIC values at 12.5-25 µg/ml.

3. Time kill analysis of PG Monthong

S. aureus; time kill analysis illustrated that at the same concentration of PG (5% PG), bactericidal activity against *S. aureus* (SP 2) and *S. aureus* ATCC 25923 was observed due to zero bacterial count within 8 and 24hr, respectively. *S. aureus* (SP 2) was more sensitive with PG according to at concentration 0.625% PG the colony counts were declined to zero by 2 hr, whereas at MIC value of PG (0.312%) zone colony count was observed in 16 hr.

E. coli; MBC value at 1.25%, PG *E .coli* (CM 49) showed more sensitive with PG than *E. coli* ATCC 25922 represented by bacterial cells was completely killed by 12 hr and 24 hr, respectively.

S. aureus showed more susceptible to PG than *E. coli*, the MIC values of *S. aureus* (SP 23, 0.312%) killed bacterial cells in 16 hr, whereas the colony count of MIC value of PG against with *E. coli* at the same concentration was not decline to zero. The bacterial isolates were more sensitive to PG than standard tested bacteria.

Antibacterial effect PG might be due to the acidic sugar, galacturonic acid, component in PG. Galacturonic acid was also found posses antibacterial activity against *S. aureus* and *E. coli* in this study.

The MBC and MIC 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. Overall results indicated that PG Monthong has potential benefit as an antimicrobial agent for treatment of cow mastitis, according to the in vitro susceptibility test in this study revealed that most of mastitis bacterial isolates were sensitive to be killed by PG.

4. A comparison of time kill analysis of bactericidal activity of PG Monthong, Chanee and native from Chumporn province

S. aureus; S. aureus was more sensitive to be killed by PG Chanee (C) and Native cultivar (Ncv) than PG Monthong (M) due to the result in table 18 and Figure 12-24 showed that at MBC of it PG at exhibit zero bacterial 0.625% count in 2, 8 and 16 hr, respectively. PG Chanee and Native cultivar showed the better bactericidal activity against *S. aureus* than PG Monthong.

E. coli; a similar resultof bactericidal activity of PG Monthong, Chanee and Native cultivar against *E. coli*. *E. coli* ATCC 25922 was killed at 1.25% PG Native cultivar within 8 hr whereas PG Monthong and Chanee show its killing effect within 24 hr and 16 hr, respectively.

In comparison the bactericidal activity of PG Montong (M), Chanee (C) and Native cultivar (Ncv) against *S. aureus* and *E. coli*, the result showed that PG Chanee and Native cultivar exhibited better bactericidal activity than PG Monthong in this study.

5. Time kill analysis of galacturonic acid

S. aureus; S. aureus (SP 23) was more sensitive with galacturonic acid than *S. aureus* ATCC 25923 when compared with the same MBC (0.312%) the colony count decline to zero in 2 hr and 24 hr. While MBC of *S. aureus* SP 2 (0.156%) produced bactericidal activity in 16 hr.

E. coli; E. coli ATCC 25922 and *E. coli* (CM 49) had the same bactericidal activity . The colony count were decline to zero in 12 hr when MBC is 2.5%.

S.aureus more sensitive with galacturonic acid than *E. coli.* It may be the difference structure of cell wall. *E. coli* had effect with large molecule than *S. aureus* because it had larger cell wall.

The gram negative strain *E. coli, K. pneumoniae and Pseudomonas* spp. had higher MBC values of galacturonic acid, mostly one douibling dilution (84.61%, 20% and 60%, respectively) than MBC values from PG Monthong. The gram positive strain *S. aureus and Streptococcus* spp. had lower MBC values, mostly three doubling dilution (88.1% and 96.7%, respectively)

It has been hypothesized that the effect of PG against bacteria may be due to their negative charge and their ability to adop amphipatic conformations. It is well known that the dual hydrophobic and hydrophilic nature of these molecules is important for their initial interaction with the bacterial membrane. Perhaps the thick peptidoglycans layer in gram positive cell envelope might impair access of the galacturonic acid to same extent to the gram positive cell membrane.

The antibacterial nature of the PG studies against gram positive is apparently related to its galacturonic acid components. The bacteriostatic of PG are suspected to be associated with the galacturonic acid content, which has been tested previously and was found to have a significant antibacterial activity. Thus, galacturonic acid which was found to be the major constituent of the investigated, is an effective antibacterial when used alone, and when combined with sugar, synergism has been observed against tested bacteria in vitro. Although the antibacterial activity of PG from durian fruit-rinds has been extensively surveyed, their antimicrobial mechanism has not been reported in great detail. Since the active antimicrobial compounds of PG are galacturonic acid, it seems reasonable that their mode of action might be similar to that of other sugars. Most of the studies on the mechanism of galacturonic acid focused on their effects on cellular membranes, altering its function and in some instances structure, causing swelling and increasing its permeability.

6. Factors effecting inhibitory activity of PG

6.1 Temperature

Effect of temperature on bactericidal activity of PG against *S. aureus* in this study showed that temperature at 25°C and 37°C was not effect killing activityof PG all bacterial cells were killed after 24 hr incubation, whereas PG bactericidal activity was reduced at incubation temperature 25°C against *E. coli* compared to incubation temperature at 37°C. The MBC value of PG was not exhibited colony count decline to zero after incubation at 37°C.

These result may be due to decreasing of reaction rate between PG and *E. coli* and reducing in the numbers of available binding sites at the *E. coli* cell surface, both being affected by low temperature. The stress of low temperature may change cell surface structures in ways that decrease the number of surface binding sites (or electronegativity) for PG.

6.2. pH or acid-base in media

S. aureus; at pH 2, MHB with PG and MHB without PG exhibited the similar inhibitory effect which was due to high acidity environment. At pH 3-5 showed higher inhibitory effect of MHB with PG more than MHB without PG, so the inhibitory activity was not only due to the acidic property of PG.

E. coli; at the same pH, MHB with PG exhibited the inhibitory effect better than MHB without PG. The inhibitory activity of PG against *E. coli* was less sensitive to pH than *S. aureus*.

6.3. Mono and divalent cations

PG in the present of 25 and 100 mM NaCl the antibacterial activity of PG against *E. coli* was decrease whereas bactericidal activity of PG against *S. aureus* was not effected. *E. coli* could be better survived in PG in NaCl than *S. aureus*. It's seemed NaCl

decrease antibacterial activity of PG against *E. coli* but NaCl did not interfere antibacterial activity of *S. aureus*.

$BaCl_2$, $CaCl_2$ and $MgCl_2$; the PG with salts concentration of 10 and 20 mM decreased the effect of PG bactericidal activity against *E. coli* but no interference bactericidal activity against *S. aureus*.

$ZnCl_2$; $ZnCl_2$ demonstrate good bactericidal activity against bacteria PG and MHB with 10 and 20 mM $ZnCl_2$ inhibited good bactericidal activity. Time kill analysis was also studied the result showed that PG and $ZnCl_2$ had the good bactericidal activity.

S. aureus; $ZnCl_2$ did not increase bactericidal activity of PG against *S. aureus* because PG without $ZnCl_2$ showed a better bactericidal activity (8hr) than the PG with $ZnCl_2$ (24 hr).

E. coli; $ZnCl_2$ demonstrated good bactericidal activity by itself, the colony counts were decline to zero in 2 hr (PG without $ZnCl_2$ the colony count decline to zero in 24 hr).

CONCLUSION

1. Agar diffusion method : *Streptococcus* spp. were the most susceptible bacteria to PG.
2. Broth microdilution test: all strains of tested bacteria showed similar susceptibility to PG. The average MBC of PG against most of the tested bacteria was 12.5-25 mg/ml.
3. The strains of *Streptococcus* spp., *Pseudomonas* spp. and *K. pneumoniae* that showed ability to survive in gentamicin (MBC $\geq$ 8 μg/ml) were found susceptible to PG with the MIC values at 12.5-25mg/ml.
4. A comparision of the antibacterial activity of PG Montong (M), Chanee (C) and Native cultivar (Ncv) against *S. aureus* and *E. coli,* indicated that PG Chanee and Native cultivar exhibited higher bactericidal activity than PG Monthong.
5. Galacturonic acid and PG of Monthong showed comparable bactericidal activity against gram negative bacteria, whereas the higher bactericidal activity of galacturonic acid than PG was observed against gram positive bacteria, *Staphylococcus* spp. and *Streptococcus* spp.
6. Time-kill study showed that *S. aureus* was more susceptible to PG than *E. coli.*
7. The effect of temperature on inhibitory activity of PG showed that inhibitory activity of PG against *S. aureus* was temperature independent (25°C and 37°C), while at 25 °C effect to decrease inhibitory activity of PG against *E. coli.*
8. At pH 3-5 PG showed higher inhibitory effect than control (HCl in MHB). PG showed higher inhibitory effect than control (HCl in MHB) in *S. aureus*. The same result was found at pH 3-4 in *E. coli.*
9. The ionic strength at 25 and 100 mM NaCl on antibacterial activity of PG, showed that NaCl decreased bactericidal effect of PG against *E. coli* but no effect against *S. aureus.*
10. The bactericidal activity of PG Montong with divalent cations, $BaCl_2$, $CaCl_2$, $MgCl_2$ and $ZnCl_2$: The PG with 10 and 20 mM $BaCl_2$, $CaCl_2$, $MgCl_2$ decreased the effect of bactericidal activity against *E. coli* but no effect against *S. aureus.*
11. PG and MHB (control) in the present of 10 and 20 mM $ZnCl_2$ showed higher bactericidal activity against *E. coli* and *S. aureus*, and *E. coli* was kill faster than *S. aureus.*