CHAPTER V

DISCUSSION &CONCLUSION

1. Phytochemical test and Susceptibility test

In recent years, there has been a rising interest in the discovery of new antimicrobial compounds due to an alarming increase in the rate of infections with antibiotic-resistant microorganisms (Davies, 1994). The search for biologically active extracts based on traditionally used plants is still relevant due to the appearance of microbial resistance to many antibiotics. In this study, alcoholic extract of *T.citrina* ROXB. showed that active constituent was tannin, which are widely known sources of these polyphenolic compounds. Several tannins with different structures inhibited microbial growth *in vitro* (Chung et al, 1998). Tannins were reported to bind to proteins primarily through multiple hydrogen bonds formed between the phenolic hydroxyl groups of tannins and the carboxyl groups of the peptide linkage of proteins (Cannon, 1966: Goldestein and Swain, 1963; Van et al, 1975). Tannins have been reported to be bacteriostatic and/or bactericidal for *Staphylococcus aureus, Streptococcus pneumonia, Bacillus anthracis, Shigella dysenteriae, and Salmonella senftenberg* (Beuchat and Heaton, 1975; Kau, 1980). Many disease-associated bacteria were affected by tannins. The growth of various diarrhea-causing pathogens was inhibited by tea extracts (Toda et al., 1989). The antimicrobial property of tannin from *T.citrina* ROXB. was also reported. (Burapadaja and Bunchoo, 1995). The results from our study showed that alcoholic extract of *T.citrina* ROXB. had inhibitory effect to all 30 strains of *E.coli.*

2. Checkerboard method

Ampicillin is readily cleaved by beta-lactamase and is useless in the treatment of infections caused by *S. aureus* or other organisms producing this enzyme. Increasing resistance has appeared in strains of *E. coli, S. pneumoniae, N. gonorrhea*, and nontyphoidal *Salmonella*. Ampicillin-sulbactam, ticarcillin-clavulanate, and piperacillin-tazobactam, which are parenteral formulations, are active against ampicillin-susceptible organisms and various ampicillin-resistant organisms, including beta-lactamase-producing strains of *E. coli*. Amoxicillin plus clavulanate is effective *in vitro* and *in vivo* for beta-lactamase – producing strains of *Staphylococci, H. influenzae*, and *E.coli* (Ball et al., 1980; Yogev et al., 1981). Therefore, this is the

first study which has been performed by checkerboard method using alcoholic extract of *T.citrina* ROXB. combined with ampicillin or with norfloxacin against 30 *E.coli* (29 ESBL-producing,1 non-ESBL-producing) strains.

The study of synergistic interaction between alcoholic extract of *T.citrina* ROXB. plus ampicillin against 30 *E.coli* strains showed the indifference activity. Therefore, the checkerboard results showed that all concentrations of ampicillin used in the combination of alcoholic extract of *T.citrina* ROXB were not the appropriate concentrations to inhibit ESBL-producing *E.coli*.

In contrary, the study of synergistic interaction between alcoholic extract of *T.citrina* ROXB. plus norfloxacin against 18 strains of ESBL-producing *E.coli* showed synergistic effect (synergistic + partial synergistic effects) from the combination of alcoholic extract of *T. citrin*a ROXB. and norfloxacin against 94.44% of the tested strains was observed. The MIC of norfloxacin was decreased by 4-fold when combined with ½ MIC of the alcoholic extract of *T. citrin*a ROXB. Thus, the MIC of norfloxacin alone, which was 64 µg/ml, was decreased to 16 µg/ml in the combined agent. This suggested that the possible mechanism of action of the plant extract was the enhancement of norfloxacin influx to the cells of ESBL-producing *E.coli*. However, the tested isolates were multidrug-resistant strains which could have more than one antimicrobial resistant mechanisms.

The results from this part of study indicated the possible use of the combination of *T.citrina* ROXB. alcoholic extract and norfloxacin in order to inhibit or kill the ESBL-producing *E.coli*

3. Time kill method

This method was performed in order to quantify the effect of the combined drug on the rate of bactericidal action at different time interval. Identical cultures are incubated simultaneously with antibiotic either alone added single or in combination with alcoholic extract of *T.citrina* ROXB. if a combination of antibiotics is more rapidly bactericidal than either drug alone, the result is termed synergism (Eliopoulos and Moellering, 1996). In this study, antibacterial activity of *T.citrina* ROXB. alcoholic extract and norfloxacin in combination at various concentrations was determined by time-kill method. The results demonstrated that alcoholic extract of *T.citrina* ROXB. had antibacterial activity against ESBL-producing *E.coli*.

The concentration of *T.citrina* ROXB. alcoholic extract used in this study was equal to 1MICs and ½MIC Thus, it was shown that the better bactericidal and bacteriostatic activities against these isolates were obtained when 1 MIC of *T.citrina* ROXB. alcoholic extract plus norfloxacin was used than when ½ MIC of the extract was used as shown as the number of cells killed, the number of strains killed and the time that the bacteria were killed.

Besides the synergistic effect of the combination of the alcoholic extract of *T. citrina* ROXB. and norfloxacin, no regrowth of the tested strains was also observed. This could possibly decrease the incidence of drug resistance as well as increase the use of norfloxacin in the treatment of infections caused by norfloxacin-resistant pathogens. In addition, the combined agent was shown to have bactericidal activity, thus, the number of bacteria killed within 24 hours was significantly higher ($p<0.05$) than those killed by norfloxacin alone. From this part of the study, it was shown that the most appropriate concentration of the alcoholic extract of *T. citrina* ROXB. in the combination with ½ MIC of norfloxacin was 10 mg/ml, since the number of bacteria killed by this combined agent after 24 hours indicated the bactericidal effect which could not be observed when using the lower concentrations of the alcoholic extract of *T. citrina* ROXB.

Interestingly, the compound(s) in *T.citrina* ROXB. alcoholic extract, possibly tannin compounds, may have antibacterial mechanism(s) that can inhibit ESBL-producing *E.coli*. Since it was found that the combined agents could shown the synergistic effect against 5 strains of *E.coli* which were resistant to amoxicillin/clavulanic acid. However, there has been no report on inhibitory effect of tannins against beta-lactamase. Therefore, tannins could cause the coagulation of various proteins (White, 1987). So they could possibly inhibit the beta-lactamase enzymes by this mechanism of action. Thus, our results suggested further studies in various aspects including the identification of active compound(s) and the *in vivo* studies on the effect of these pure compounds to obtain more conclusive evidence.

Conclusion

The results obtained suggested that antibacterial activity of the combination extract plus norfloxacin were higher than the antibacterial activity of each drug. It is concluded that the combination of extract plus norfloxacin could be promising

alternatives in the treatment of infections due to ESBL-producing *E.coli* that were resistant to ampicillin and norfloxacin.

Preliminary informations obtained from this study indicated that the alcoholic extract of *T.citrina* ROXB. was a very attractive interesting crude drug for the discovery of the new drug and might be used in combination with the antimicrobials. However, it would also be important to test the action of *T.citrina* ROXB. alcoholic extract on a larger range of antibiotic-resistant bacteria, and undergo further pharmacological evaluation to gain further insight into the specificity of the antibacterial activity of the pure compound(s) from the alcoholic extract of *T. citrina* ROXB. in order to obtain valuable scientific data concerning the use of Thai medicinal plant.