# CHAPTER III

# MATERIALS & METHODS

## 1. Microorganisms

The bacterial strains used throughout this study were 29 strains of Extended spectrum beta-lactamases producing *Escherichia coli* and 1 strain of non- Extended spectrum beta-lactamases producing *Escherichia coli.* These bacteria were clinically isolated from the patients at Siriraj Hospital and where tested for ESBLs production were performed by Taipobsakul (2005). *Escherichia coli* ATCC 25922 was used as the control strain.

## 2. Chemicals

Standard powder of ampicillin was kindly provided by Siam Bheasach, Thailand and standard powder of norfloxacin was purchased from Sigma (U.S.A). Working standard solutions were prepared immediately prior to use, as recommended by the manufacturers before dilute with test broth.

Antimicrobial disks were ampicillin (10 μg), amoxicillin/clavulanic acid (20/10 μg ) and norfloxacin (10 μg) , which were purchased from BBL chemicals. (U.S.A)

Dimethy sulfoxide (DMSO), Acetic acid (glacial GR.Grade), Ethanol 95% and Nitrocefin disk were purchased from Sigma, St. Louis (U.S.A.), E.Merck (Germany), Government Pharmaceutical Organization, Bangkok and BBL chemicals (U.S.A), respectively

## 3. Medicinal plant materials

The fruit of *Terminalia citrina* ROXB. was purchased from well known Thai Herb Pharmacy "Chao-Krom-Per", Bangkok, Thailand in 2005. The plant materials were identified by comparison with the specimen in the Museum of Natural Medicine, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Thailand.

3.1 Preparation of crude extract

3.1.1 The ripe fruit of the plant was crushed with an electric blender.

3.1.2 The powder was macerated with 95% ethanol 1,700 ml for 3 days and 70% ethanol 1,550 ml for 3 days.

3.1.3 The solvent was then filtered using Whatman No. 1 filter paper and concentrated using a Rotatory evaporator at $40^{0}C$.

3.1.4 The extract 1,000 g. of starting plant material gived 260.24 g. of dry extracted

3.1.5 The concentrated ethanol extract has been kept in the desicator and will be dissolved in 2% dimethy sulfoxide (DMSO, Sigma, St. Louis, USA) before use.

3.2. Phytochemical screening

Chemical tests were carried out on the ethanol extract using standard procedures to identify the constituents as described by Sofawara (1993), Trease and Evans (1989) and Harborne (1973).

3.2.1 Test for tannins

0.5 g. of the dried powdered samples were boiled in 20 ml of water in a test tube and then filtered. The sample was divided into 3 portions, which the first portion was use as the control. A few drops of 1% gelatin solution were dropped into the second portion of the sample. A white turbid precipitate colouration was observed indicating the presence of tannins. A few drops of 0.1% ferric chloride solution was added into the third portion. A brownish green or blue-black colouration was observed indicating the presence of tannins.

3.2.2 Test for flavonoids

0.5 g. of the dried powdered samples were dissolved in 50% ethanol and then filtered. The sample was divided into 2 portions, which the first portion was used as the control sample. One to two drops of concentrated HCl, and 2-3 pieces of magnesium ribbons were added to the second portion (Kapoor et al., 1969). A pink to red colouration in extract within 1-2 minutes was observed indicating the presence of flavonoids.

4. Detection of beta-lactamase.

The cefinase disk were used in this study. It is the paper disk which is impregnated with chromogenic cephalosporin, nitrocefin. This compound exhibits a very rapid colour change from yellow to red as the amide bond in the beta-lactam ring is hydrolyzed by a beta-lactamase. When the bacterium produces this enzyme in significant quantities, the yellow-coloured disk turns red in the area where the isolate is smeared. Each disk is used to test one bacterial strain for the presence of beta-lactamase.

4.1 Nitrocefin-based test

The well-isolated colony of each 18 hours cultures of the clinical isolates and control strain of *E.coli* were selected from Tryptic soy agar (TSA) plates. 20 μl of strile water was dropped to cefinase disks. The top of 1-2 well-isolated colonies were touched with a loop and tranfered on cefinase disks. The beta-lactamase activity was observed within 5 min.

4.2 Results and Interpretation

A positive result will show a yellow to red colour change on the area where the culture was applied. A negative result will show no colour change on the disk.

5. Antibiotic susceptibility test (NCCLS, 2004)

Paper disk susceptibility test was performed according to the Kirby-Bauer method by NCCLS (NCCLS, 2004). *E.coli* ATCC 25922 was also included in this study as the control strain. The susceptibility patterns of all 30 isolates against all the tested antimicrobial agents were determined.

5.1 Preparation of media

5.1.1 Mueller – Hinton agar (MHA) was prepared from a commercially available dehydrated base according to the manufacture's instructions.

5.1.2 Immediately after autoclaving, the media was allowed to cool in 45 °C water bath.

5.1.3 The freshly prepared and cooled medium was poured into glass, flat-bottomed petri dishes on a level, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 25 ml for plates with a diameter of 100 mm.

5.1.4 The agar medium should be allowed to cool at room temperature and all prepared plates must be examined sterility by incubating at 37 °C for 24 hours.

5.1.5 Unless the plates were used within the same day, they were stored in a refrigerator (2 to 8 °C) and should be used within 7 days after preparation.

5.2 Alcoholic extract of *T.citrina* ROXB. disk preparation

5.2.1 20 μl of extract concentrations 1.0, 2.5 and 5.0 mg/ml, respectively, were dropped on paper disk (Whatman no.1, 6 mm.)

5.2.2 These paper disk were left in a sterile petri dishes until the solvent was completed dried at room temperature before use.

5.3 Inoculum Preparation

5.3.1 The well-isolated colony of each 18 hours *E.coli* from clinical specimen and *E.coli* ATCC 25922 were selected from Tryptic soy agar (TSA) plates and transferred to a tube containing 5 ml normal saline solution (NSS).

5.3.2 The suspension was adjusted to match the turbidity of the 0.5 Mcfarland standard solution. This result in a suspension containing approximately 1 to 2 x $10^8$ CFU/ml.

5.4 Inoculation Test Plates

5.4.1 Optimally, within 15 minutes after adjusting the turbidity of the inoculum suspension, a sterile cotton swab was dipped into the adjusted suspension. The swab should be rotated several times and pressed firmly on the inside wall of the tube above the fluid level. This will remove excess inoculum from the swab.

5.4.2 The dried surface of an agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated by streaking two more times, rotating the plate approximately 60 ° each time to ensure an even distribution of inoculum.

5.5 Application of Disks to Inoculated Agar Plates

5.5.1 The antibiotic disks and alcoholic extract of *T.citrina* ROXB. disks were applied to the surface of the medium with sterile forceps. Each disk must be pressed down to ensure complete contact with the agar surface. They must be distributed evenly so that they are no closer than 24 mm. from center to center. Because some of the drug diffuses almost instantaneously, a disk should not be

relocated once it has come into contact with the agar surface. Instead, place a new disk in another location on the agar.

5.5.2 The plates were inverted and placed in ambient air incubator set to 37 °C within 15 minutes after the disks were applied in ambient air

5.6 Reading Plates and Interpreting Results

5.6.1 The diameter of each zone of inhibition was measured with digital sliding venier caliper.

5.6.2 The size of the inhibition zone were interpreted by referring to the NCCLS, 2004 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have been tested (Tables 3-1)

Table 3-1 Zone diameter interpretive standards breakpoints for *E.coli* and *E.coli* ATCC 25922 (NCCLS,2004)

| Drug | Disk content | Zone diameter (mm) | | | |
|---|---|---|---|---|---|
| | | *E.coli* | | | *E.coli* |
| | | R[a] | I[b] | S[c] | ATCC25922 |
| Ampicillin | 10 µg | ≤13 | 14-16 | ≥17 | 16-22 |
| Amoxicillin/clavulanic acid | 30 µg | ≤13 | 14-17 | ≥18 | 19-25 |
| Norfloxcin | 10 µg | ≤12 | 13-16 | ≥17 | 28-35 |
| imipenem | 10 µg | ≤13 | 14-15 | ≥16 | 26-32 |

[a]Resistant, [b]intermediate, [c]Susceptible

6. Agar dilution MIC determinations (NCCLS, 2004)

Agar dilution method was performed according to NCCLS (NCCLS, 2004). All isolates and *E.coli* ATCC 25922 were determined minimum inhibitory concentration (MIC) of ampicillin, norfloxacin.and alcoholic extract of *T.citrina* ROXB.

6.1 Preparation of agar dilution plates

6.1.1 The two-fold dilution of ampicillin solution ( 0.03-256 µg/ml) , norfloxacin (0.03-256 µg/ml) and alcoholic extract of *T.citrina* ROXB. (0.3-40 mg/ml) were prepared. Thus the final volume in each plate consisted of 2.5 ml of each dilution of the antimicrobial agents or plant extract and 22.5 ml of MHA, so the stock solutions of each agent were prepared to be ten-fold greater than the desired final concentrations.

6.1.2 MHA was prepared from a commercially available dehydrated base according to the manufacturer's instructions.

6.1.3 Immediately after autoclaving, allow it to cool in a 55 °C water bath and then pipetted 2.5 ml of each dilution of the test agents into 22.5 ml of MHA.

6.1.4 The agar and antimicrobial agent solution were mixed thoroughly and then pour into plates.

6.1.5 The agar dilution plates were allowed to solidity at room temperature, and used immediately.

6.2 Inoculum preparation

6.2.1 The agar plates were marked for orientation of the inoculum spots.

6.2.2 A 1 µl of each inoculum was applied to the agar surface by the use of an inocula-replcating device. The final inoculum on the agar will then be approximately $10^4$ CFU per spot.

6.2.3 A growth-control plate (no antimicrobial agent) was inoculated first and then, starting the lowest concentration, the plates containing the different concentrations were inoculated.

6.3 Incubating agar dilution plates

The incubated plates were allowed to stand at room temperature until the moisture in the inoculum spots have been absorbed into the agar until the spots were dried, but no more than 30 minutes. The plates were inverted and incubated at 37 °C for 24 hours.

6.4 Determining agar dilution end points

6.4.1 The MICs were recorded as the lowest concentration of antimicrobial agent that completely inhibited the growth, disregarding a single colony or a faint haze caused by the inoculum.

6.4.2 The MICs were interpreted by referring to the NCCLS, 2004 and the organisms were reported as either susceptible, intermediate, or resistant to the agents that have tested (Table 3-2)

Table 3-2 MICs interpretive standards breakpoints (μg/ml) (NCCLS,2004)

| Drug | Minimum Inhibitory Concentration [MICs] (μg/ml) | | | |
|---|---|---|---|---|
| | *E.coli* | | | *E.coli* |
| | R[a] | I[b] | S[c] | ATCC 25922 |
| ampicillin | ≥32 | 16 | ≤8 | 2-8 |
| norfloxacin | ≥16 | 8 | ≤4 | 0.03-0.12 |

[a]Resistant, [b]intermediate, [c]Susceptible

7. Checkerboard synergy testing.

The checkerboard microdilution panel method served to determine the activity of alcoholic extract of *T.citrina* ROXB. in combination with ampicillin and norfloxacin. The concentrations tested for ampicillin were 4, 8, 16, 32, 64, 128, 256 and 512 μg/ml, norfloxacin were 1, 2, 4, 8, 16, 32, 64, 128 μg/ml. and for alcoholic extract of *T.citrina* ROXB. were 0.3125, 0.625, 1.25, 2.5, 5, 10, 20, 40 mg/ml.

7.1 The twofold dilutions of drug or the extract were prepared volumetrically in the broth. The final volume of 200μl in each well consisted of 50μl of MHB , 50μl of broth for drug , 50μl of broth for extract and 50μl of broth containing a suspension of the organism was obtained. Thus antimicrobial concentrations used in the initial (stock) solutions were prepared four-fold in greater than the desired final concentration. The concentrations tested for each antimicrobial and extract typically ranged from 5 dilutions below the MIC to twice the MIC or higher.

7.2 A series of antimicrobial and extract solutions containing four times the desired final concentrations were taken to produce the desired range of drug concentration by adding an aliquot of those solution to each well in the appropriate row or column (as shown in Figure 3-1)

| Extract ↑ | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| 40 | 40/1 | 40/2 | 40/4 | 40/8 | 40/16 | 40/32 | 40/64 | 40/128 |
| 20 | 20/1 | 20/2 | 20/4 | 20/8 | 20/16 | 20/32 | 20/64 | 20/128 |
| 10 | 10/1 | 10/2 | 10/4 | 10/8 | 10/16 | 10/32 | 10/64 | 10/128 |
| 5 | 5/1 | 5/2 | 5/4 | 5/8 | 5/16 | 5/32 | 5/64 | 5/128 |
| 2.5 | 2.5/1 | 2.5/2 | 2.5/4 | 2.5/8 | 2.5/16 | 2.5/32 | 2.5/64 | 2.5/128 |
| 1.25 | 1.25/1 | 1.25/2 | 1.25/4 | 1.25/8 | 1.25/16 | 1.25/32 | 1.25/64 | 1.25/128 |
| 0.625 | 0.625/1 | 0.625/2 | 0.625/4 | 0.625/8 | 0.625/16 | 0.625/32 | 0.625/64 | 0.625/128 |
| 0.3125 | 0.3125/1 | 0.3125/2 | 0.3125/4 | 0.3125/8 | 0.3125/16 | 0.3125/32 | 0.3125/64 | 0.3125/128 |
| 0 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 |

→ Drug

Figure 3-1 Checkerboard technique. In the checkerboard, serial dilution of drug and extract are performed using drug and extract proportional to MICs of the drug and extract being tested. (Modified from Eliopoulos and Moellering, 1996)

7.3 The interpretations of the antimicrobial combination interactions were done by reading the first clear well in each row of the panel with both agents. Based on this reading, the result of checkerboard study were interpreted by the pattern they form on the isobologram (figure 3-2)

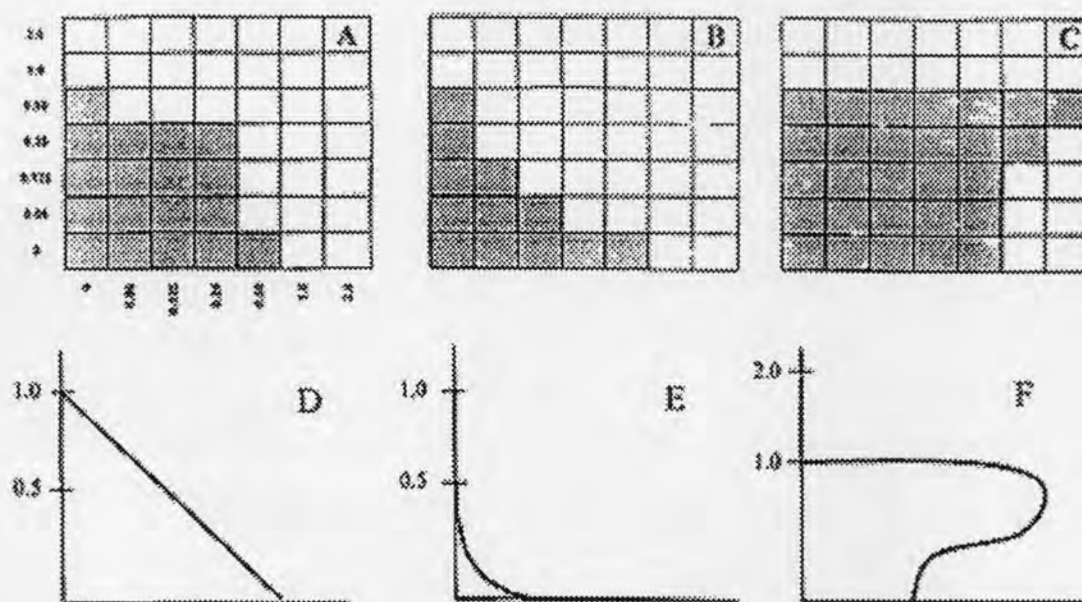


Figure 3-2 Assessment of antimicrobial combinations with the checkerboard method. A, B, and C. Results of testing combinations of drug and extract. Shading, visible growth. Concentrations are expresses as multiples of MIC. Isobolograms (plotted on an arithmetic scale) that represent the results of checkerboards shown in D,E, and F, respectively. A and D. Additive effect. B and E. Synergism. C and F. Antagonism. (Modified from Eliopoulos and Moellering, 1996)

7.3.1 To evaluate the effect of the combinations, the fractional inhibitory concentration were calculated for each antimicrobial alone and in combination. The following formular were used to calculate the FIC.

$$\text{FIC of antimicrobials} = \frac{\text{MIC of antimicrobials in combination}}{\text{MIC of antimicrobials alone}}$$

$$\text{FIC of extract} = \frac{\text{MIC extract in combination}}{\text{MIC of extract alone}}$$

$$\text{FIC index } (\Sigma\text{FIC}) = \text{FIC of antimicrobials} + \text{FIC of extract}$$

7.3.2 FIC index results for each combination were defined as:

7.3.2.1 synergy, if the decrease in the MIC of each agent was ≥4-fold ($\Sigma$FIC ≤ 0.5)

7.3.2.2 partial synergy, if the decrease in the MIC of 1 agent was ≥4-fold and the decrease in the MIC of the other agent was 2-fold ($\Sigma$FIC, >0.5 and <0.1).

7.3.2.3 additive, if the decrease in the MIC of both agent was 2-fold ($\Sigma$FIC = 1).

7.3.2.4 indifference, if the interaction did not meet the above criteria and were not antagonistic ($\Sigma$FIC,>1 and <4).

7.3.2.5 antagonism, if an increase in the MIC of both agents was ≥4-fold ($\Sigma$FIC ≥ 4).

The smallest FIC value was used to establish the antimicrobial combination interaction for each specific strain, except for antagonism, which was preferably reported. Results were expressed as percentage of isolates with synergism, additive, indifference and antagonism.

8. Time kill assays

The antibacterial activity of the combination was performed according to the time kill method by Elipoulos and Moellering, 1996. Drug concentration used for the time kill assays were based on criteria (i) concentration likely to produce synergy. partial synergy and additive as seen in checkerboard testing (ii) concentration that were no more than of each drug.

8.1 Alcoholic extract of *T.citrina* ROXB. and antimicrobials concentrations used in initial (stock) solutions were prepared four fold, two fold greater than desired final concentration, respectively.

8.2 A 5 ml of each alcoholic extract of *T.citrina* ROXB. and antimicrobials were pipetted into Mueller Hinton broth (MHB) for prepared working media adding the standardized inoculum (Final volume of working media = 5 ml). As the result, there had been 6 groups of control (no antimicrobial agents), extract ½ MIC alone, extract 1 MIC alone, antimicrobials ½ MIC alone, extract ½ MIC combined with antimicrobials ½ MIC and extract 1 MIC combined with antimicrobials ½ MIC.

8.3 Inoculum which was adjusted to match the turbidity of the 0.5 McFarland standard solution, contained approximately 1 to 2 x $10^8$ CFU/ml. was then diluted ten fold to make 1 to 2 x $10^7$ CFU/ml. of the bacterial inoculum.

8.4 A 5 ml of inoculum was pipetted into the working media and incubated at 37 °C in shaking water bath.

8.5 The samples were collected for culture at the time 0, 2, 4, 6, 8 and 24 hours after the microorganism was exposed to in each group of the antimicrobials including the control group. A 0.5 ml of the collected sample was diluted ten fold in NSS and 20 μl of each dilution was dropped on TSA plates which were then incubated at 37 °C for 16-18 hours.

8.6 The quantity of survival bacteria in each group was calculated to obtain the killing curves data. The quantity of survival bacteria in each group was calculated to obtain the killing curves data. Killing curve were constructed by Microsoft Excel 2002 at each time interval. The $\log_{10}$ change of the viable cell counts compared to the starting inoculum was determined.

8.6.1 The results were analyzed by determining the number of strains which yield changes in the $\log_{10}$ number of CFU/ml of -1,-2 and -3 at 2,4,6,8 and 24 hours compared to the counts at 0 hours. A given concentration of antimicrobial alone or in combination was considered bactericidal of it reduced the original inoculum size by $\geq 3 \log_{10}$ CFU/ml ($\geq$ 99.9% killing) at each of the time periods or bacteriostatic if the inoculum size was reduced by 0-3 $\log_{10}$ CFU/ml. The regrowth was defined as an increase of $\geq$ 2 log CFU/ml after $\geq$6 hours. (Amsterdam, 1996; Pankuch, Jacobs and Appelbaum, 1994; Satta, et al., 1995).

8.6.2 The quantitative evaluation of antimicrobial effect was calculated as in the published article (Firsov, et al., 1997).

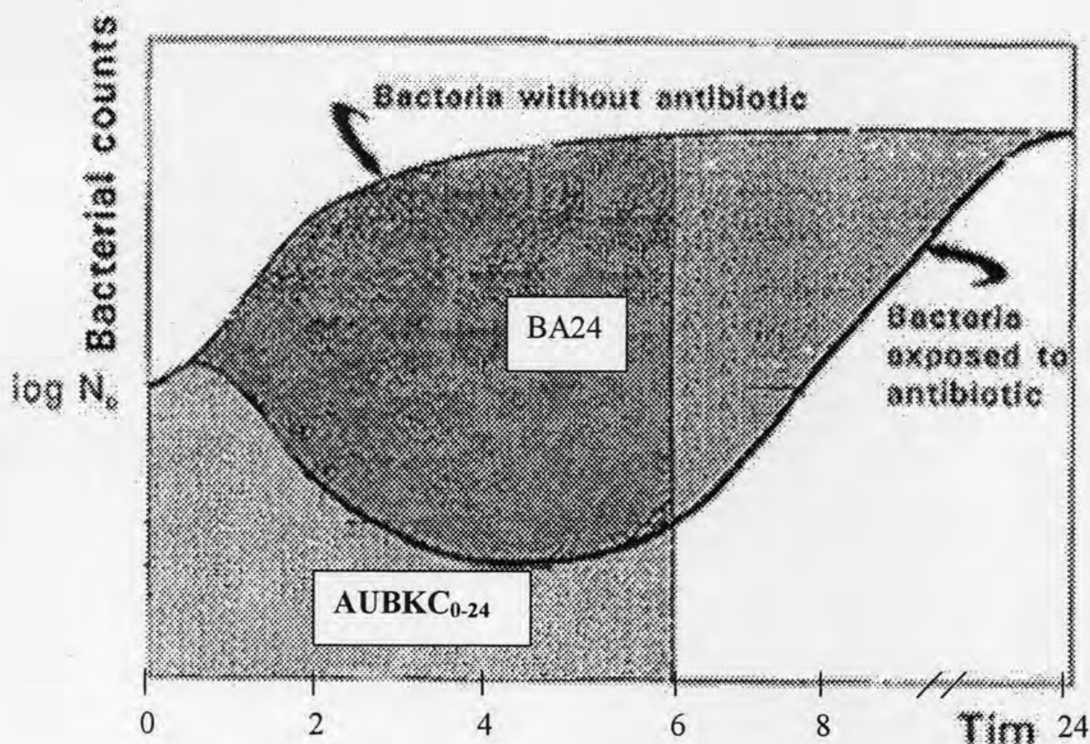


Figure 3-3 Parameters for quantifying bacterial killing and regrowth curve and the antimicrobial effect. (Modified from Firsov, et al., 1997)

The following parameters were calculated by various methodologies as follow:

$AUBKC_{0-24}$ = Area under the bacterial killing and regrowth curves that were calculated by the trapezoidal rule for 24 hours.

Bacteriolytic area for 24 hours (BA24) = the area between control growth curve and the bacterial killing and regrowth curves ($AUBKC_{0-24}$ of the control growth curve substracted by $AUBKC_{0-24}$ of the bacterial killing and regrowth curves)

Statistic analysis

Student's t-test was used to compared the Log change of viable cell counts, $AUBKC_{0-24}$ and $BA_{24}$, which expressed their mean value (± SD) values. Any value of P below 0.05 was considered as significant.