CHAPTER IV

RESULT

1. Phytochemical screening test

This study carried out on the alcoholic extract of *T.citrina* Roxb. revealed the presence of medicinally active constituents. The phytochemical characters of the *T.citrina* Roxb. investigated are summarized in Table 4-1. Tannin was present in this plants but alkaloids and flavonoid were not found in this plant.

Table 4-1 Qualitative analysis of the phytochemicals of the medicinal plant.

Plants	Alkaloids	Tannin	Flavonoid
*T.cit*rina ROXB.	-	+	-

+ = Presence of constituent, - = Absence of constituent

2. Detection of beta-lactamase activity (Raw data were shown in Table A-1 in Appendices.)

From nitrocefin-base test, all 30 strains of *E.coli* presence a positive reaction indicated that all the tested microorganisms produced beta-lactamase. (Figure 4 -1)

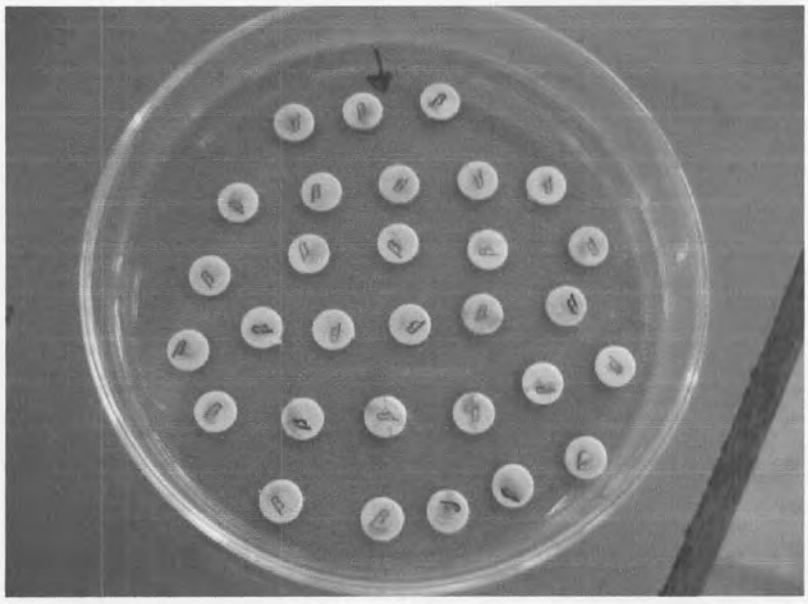

Figure 4-1 All disks turned brown-red (positive)

3. Susceptibility test

3.1 Disk diffusion method

Consequently, in order to detect a potential antimicrobial activity in alcoholic extract of *T.citrina* Roxb. against 30 strains of *E.coli* the disk diffusion method was performed The maximal zones of inhibition ranged from 7.13 - 10.01 mm. by the concentration of alcoholic extract of *T.citrina* Roxb. at 0.1 mg/disk but not at the concentration 0.02 mg/disk and 0.05 mg/disk, respectively. (Table A-2 in Appendices) Inhibitory activities with minimum inhibitory concentration (MIC) in alcoholic extract of *T.citrina* Roxb. against 30 strains of *E.coli* by agar dilution method were 10 mg/ml. (Table A-3 in Appendices.)

From disk diffusion test according to NCCLS, all *E.coli* isolates were resistant to ampicillin, while 53.3% were resistant to amoxicillin/clavulanic acid and 56.66% to norfloxacin (Table 4-2) [Table A-4 in Appendices]. All *E.coli* were susceptible to imipenem while only 40% were susceptible to norfloxacin (Table 4-2). The distribution of ampicillin resistance of all *E.coli* was shown in Figure 4-2. 6 strains of *E.coli* (20%) were resistant to ampicillin, 7 strains (23.33%) were resistanct to both ampicillin and amoxicillin/clavulanic acid, 8 strains (26.66%) were resistant to ampicillin and norfloxacin, and 9 strains (30%) were resistant to the three antimicrobials: ampicillin, amoxicillin/clavulanic acid and norfloxacin.

Table 4-2 *In vitro* activity of ampicillin, amoxicillin/clavulanic acid, norfloxacin and imipenem against 30 strains of *E.coli* as tested by disk diffusion method.

Drug	No. of isolates (% susceptibility)		
	Resistant	Intermediate	Susceptible
Ampicillin	30 (100)	0	0
Amoxicillin/clavulanic acid	16 (53.33)	10 (33.33)	4 (13.33)
imipenem	0	0	30 (100)
norfloxacin	17 (56.66)	1 (3.33)	12 (40)

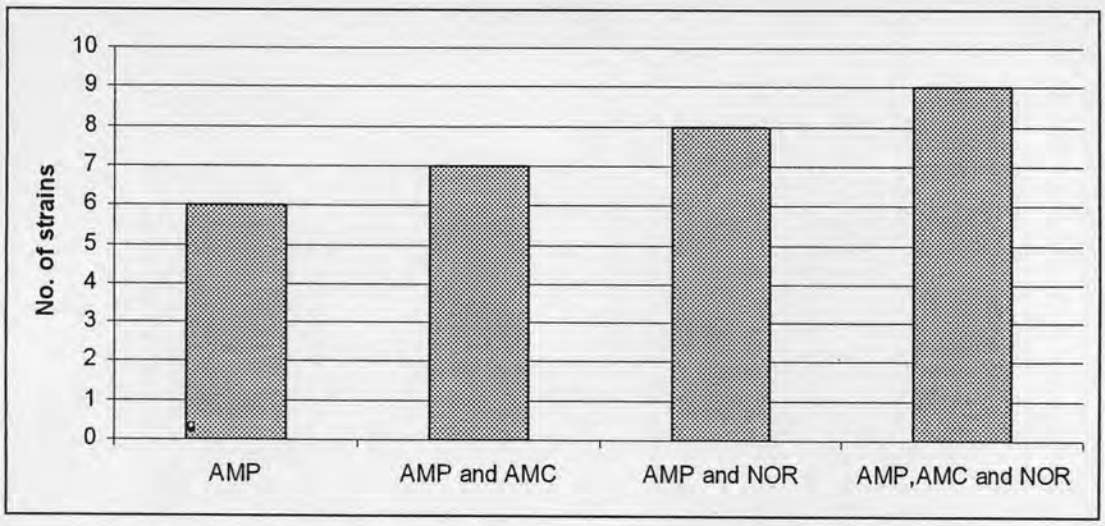

Figure 4-2 Distribution of resistance strains of *E.coli*

3.2 Agar dilution method

The range of MICs observed, as well as the MIC_{50}, MIC_{90} and percentage of susceptible strains of ampicillin and norfloxacin among the 30 isolates were shown in Table 4-3. Ampicillin had no activity against all strains tested. MICs of ampicillin ranged from 256 to >256 μg/ml which were the high level of resistance (susceptibility breakpoint ≤8 μg/ml). The MIC_{50}, MIC_{90} of ampicillin were >256μg/ml. Slightly less than one half (40%) of the strains were susceptible to norfloxacin (susceptibility breakpoint ≤4 μg/ml).

MICs of norfloxacin against 11 susceptible strains *E.coli* were 0.03-2 μg/ml and against 18 resistance strains *E.coli* were 32-64 μg/ml. The percent susceptibility of the tested organisms to norfloxacin was slightly different when the results from disk diffusion method (Table 4-2) was compared with those from agar dilution method (Table 4-3) [Zone diameter interpretive standards breakpoints of norfloxacin are shown as followed: resistance≤12 mm; intermediate 13-16 mm. and susceptible ≥ 17 mm.]. One strain (strain no. P2) was intermediate susceptible to norfloxacin as tested by disk diffusion method (zone size = 13.23 mm.) but was resistant to norfloxacin as tested by agar dilution method (MIC = 32 μg/ml). (Raw data of susceptibility testing by disk diffusion method and agar dilution method were shown in Table A-4 in Appendices.)

Table 4-3 *In vitro* activity of ampillin and norfloxacin against 30 strains of *E.coli* as tested by agar dilution method.

	MICs (μg/ml)			No. of isolates (% susceptibility)		
	Range	MIC_{50}	MIC_{90}	Resistant	Intermediate	Susceptible
Alcoholic extract of *T.citrina*	10- 10*	10	10	ND	ND	ND
ampicillin	256 - >256	>256	>256	30 (100)	0	0
norfloxacin	0.03 - 64	32	64	18 (60)	0	12 (40)

* unit of MIC = mg/ml, ND = not determine

4. Synergist test (Raw Data of checkerboard were shown in Figure A-1 to A-30 of ampicillin plus extract. and A-31to A-48 of norfloxacin plus extract in Appendices)

Checkerboard method was used to assess the MIC and the synergistic activity of two antimicrobial agent combinations including alcoholic extract of *T.citrina* ROXB.plus ampicillin against 30 strains of *E.coli* (29 strains were ESBL-producing *E.coli* and 1 strain was non-ESBL-producing *E.coli*); alcoholic extract of *T.citrina* ROXB.plus norfloxacin against 18 strains of *E.coli* (all of 18 strains were ESBL-producing *E.coli*). The MICs of all single drugs were higher than the resistance level in the interpretive guidelines from NCCLS, 2004.

The synergistic interactions between alcoholic extract of *T.citrina* ROXB. plus norfloxacin in this study were not only assessed from the MIC value but also were evaluated from the graph shape plotted on the isobologram and the fractional inhibitory concentration (FIC) index that were modified from checkerboard result as described in chapter III (method section). The graph shape of alcoholic extract of *T.citrina* ROXB.plus ampicillin in tested 30 strains of *E.coli* were in linear (Figure 4-3) and was defined as the indifference effect (FIC index = 2). whereas, the graph shape of alcoholic extract of *T.citrina* ROXB. plus norfloxacin in tested 18 strains of ESBL-producing *E.coli* were in the concave curve and were defined as synergism effect in 11 strains (shown in Figure 4-4 to Figure 4-6), partial synergism effect in 6 strains (33.33%) (shown in Figure 4-7), and the straight curve (additive pattern) was displayed in 1 strain (5.55 %) (shown in Figure 4-8).

Table 4-4 Effect of the combination of alcoholic extract *T.citrina* ROXB. plus ampicillin against 30 strains of ampicillin resistant -*E..coli* and alcoholic extract of *T.citrina* ROXB. plus norfloxacin against 18 strains of norfloxacin resistant -*E..coli* by checkerboard method

Effect	Combination [number (%) of isolates]	
	Alcoholic extract *T.citrina* ROXB. plus ampicillin	Alcoholic extract of *T.citrina* ROXB. plus norfloxacin
Synergy	-	11 (61.11)
Partial synergy	-	6 (33.33)
Additive	-	1 (5.55)
Indifference	30 (100)	-

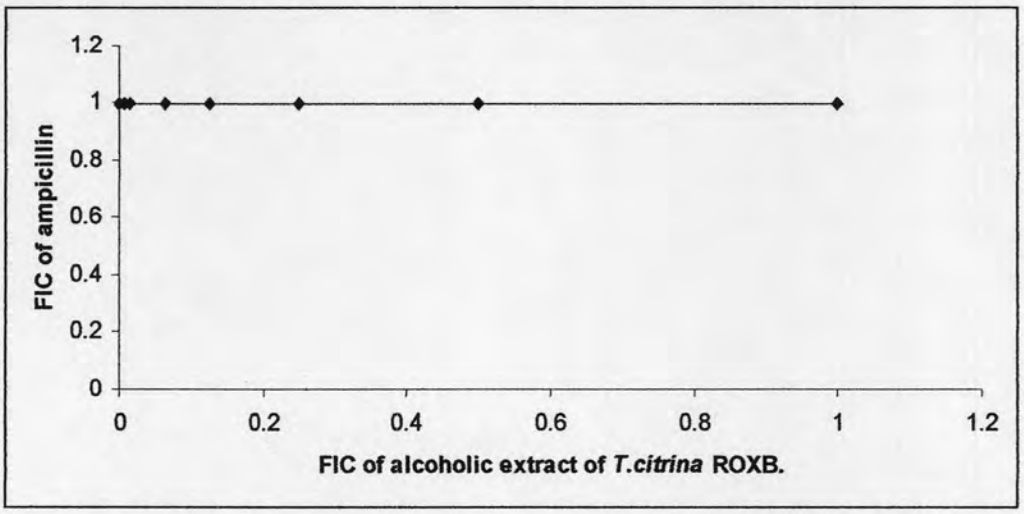

Figure 4-3 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus ampicillin combinations against 30 strains of *E.coli*

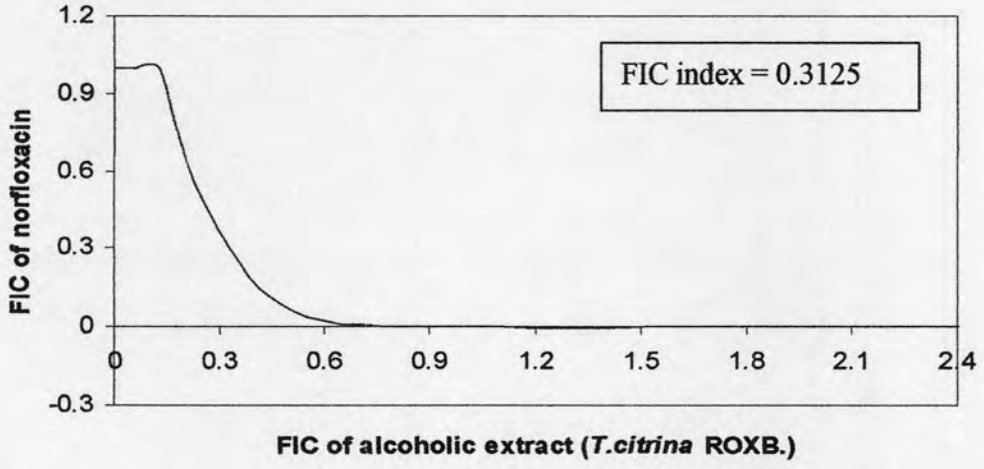

Figure 4-4 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus norfloxacin combinations against *E.coli* strain no.U25, P18

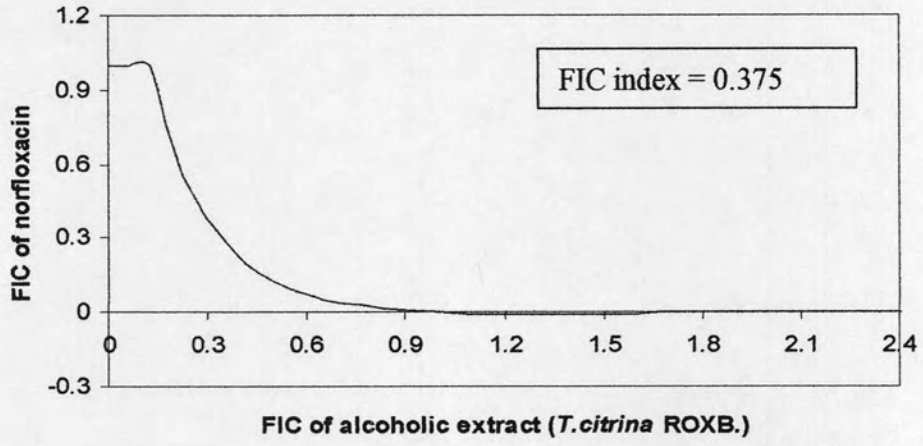

Figure 4-5 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus norfloxacin combinations against *E.coli* strain no.U1, U34, B23, B25, P12, U16, U19

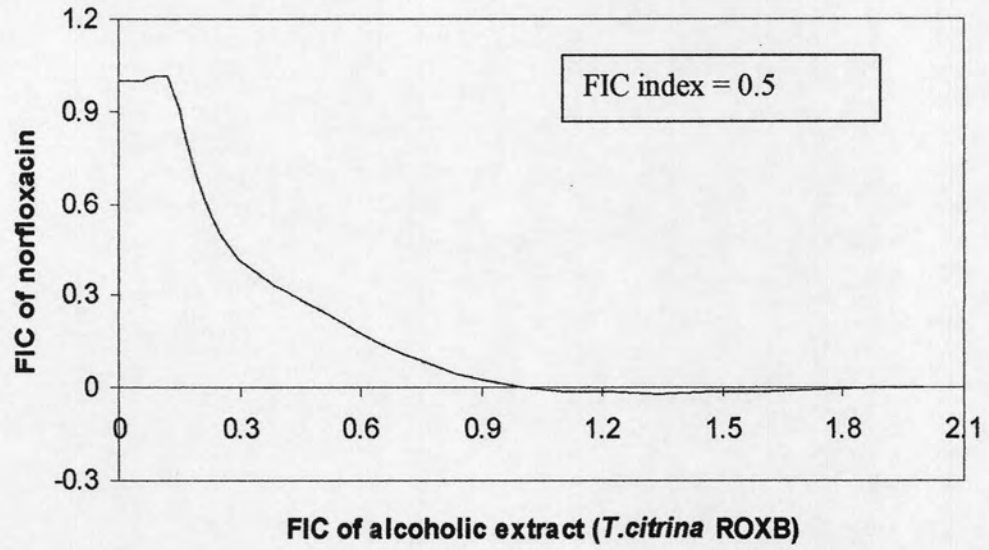

Figure 4-6 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus norfloxacin combinations against *E.coli* strain no.P2, P17

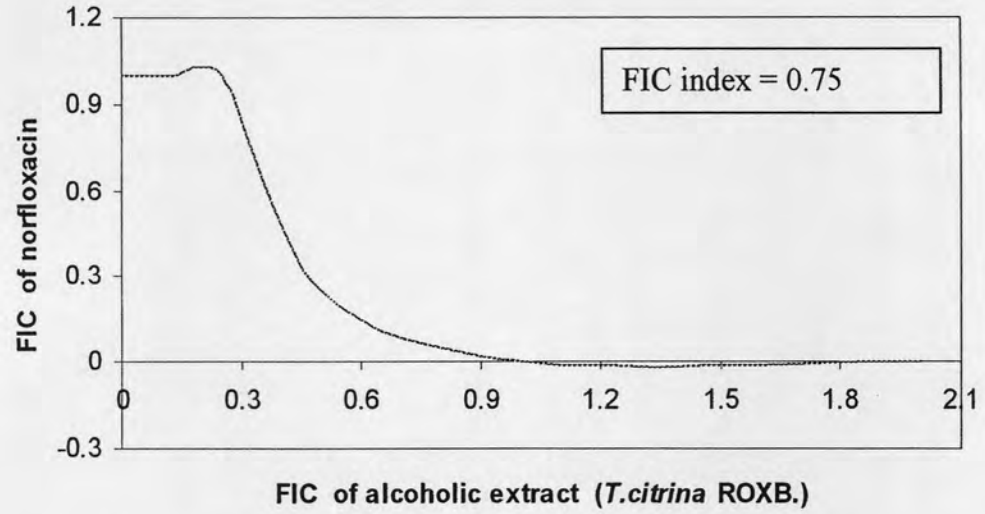

Figure 4-7 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus norfloxacin combinations against *E.coli* strain no.U8, U38, U43, U51, U56, B12

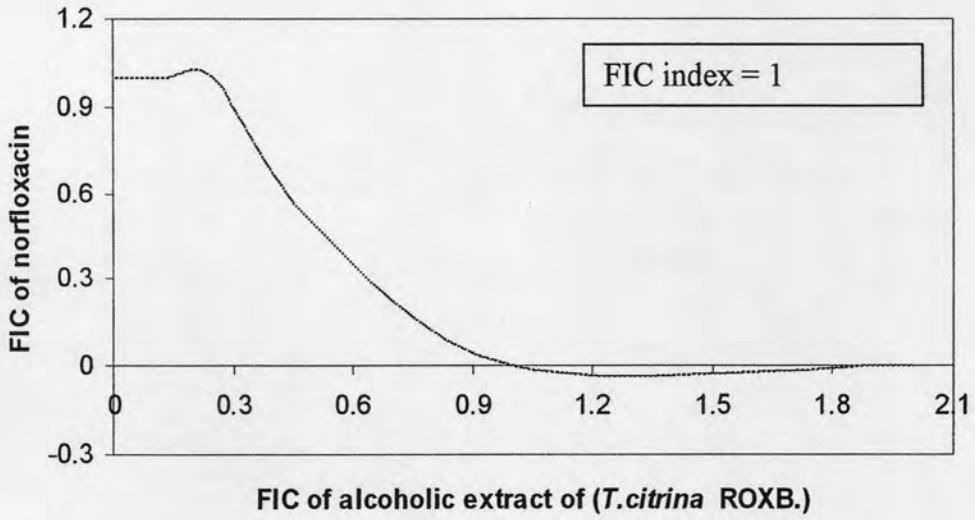

Figure 4-8 The isobologram of alcoholic extract (*T.citrina* ROXB.) plus norfloxacin combinations against *E.coli* strain no.U10

The FIC index calculated from alcoholic extract of *T.citrina* ROXB.-ampicillin combination to 29 strains were ESBL-producing *E.coli* and 1 strain was non-ESBL-producing *E.coli* is 2. Nevertheless, the FIC index calculated from alcoholic extract of *T.citrina* ROXB.-norfloxacin in tested 18 strains of ESBL-producing *E.coli* were equal, lower and high than 0.5 (0.3125, 0.375, 0.5, 0.75 and 1, respectively). [Table 4-5]

Table 4-5 Results obtained with antibiotic combinations by checkerboard method

No.	Strain no.	extract-ampicillin		extract-norfloxacin	
		FIC index	Interpretation[a]	FIC index	Interpretation[a]
1.	U1	2	I	0.375	S
2.	U3	2	I	ND	ND
3.	U5	2	I	ND	ND
4.	U6	2	I	ND	ND
5.	U8	2	I	0.75	P
6.	U10	2	I	1	A
7.	U16	2	I	0.375	S
8.	U19	2	I	0.375	S
9.	U21	2	I	ND	ND
10.	U25	2	I	0.3125	S
11.	U34	2	I	0.375	S
12.	U38	2	I	0.75	P
13.	U43	2	I	0.75	P
14.	U51	2	I	0.75	P
15.	U56	2	I	0.75	P
16.	U59	2	I	ND	ND
17.	B3	2	I	ND	ND
18.	B6	2	I	ND	ND
19.	B9	2	I	ND	ND
20.	B12	2	I	0.75	P
21.	B23	2	I	0.375	S
22.	B25	2	I	0.375	S
23.	B27	2	I	ND	ND
24.	P2	2	I	0.50	S
25.	P5	2	I	ND	ND
26.	P7	2	I	ND	ND
27.	P12	2	I	0.375	S
28.	P17	2	I	0.50	S
29.	P18	2	I	0.3125	S
30.	P23	2	I	ND	ND

[a] S = synergy, P= partial synergy, A = additive, I = indifference ; extract = alcoholic extract of *T.citrina* ROXB
ND = not determine (12 strains were susceptible to norfloxacin)

5. Time kill studies (Raw data were shown in Appendices Table A-5 to A-6)

The antibacterial activity of combination of alcoholic extract of *T.citrina* ROXB. plus norfloxacin against 18 strains of ESBL-producing *E.coli* tested by time kill method. The mean $\log_{10}$ decrease of viable cell counts and bacteriolytic area for 24 hours (BA_{24}) by the combination of extract of *T.citrina* ROXB. plus norfloxacin were shown in Figure 4-9 and Table 4-6. The combination of ½ MIC of extract plus ½ MIC of norfloxacin were shown bactericidal activity because ≥3 log CFU/ml of the bacteria

were reduced during the first 4 hour of growth. The combination of 1 MIC of extract plus ½ MIC of norfloxacin were shown bactericidal activity during the first 2 hour of growth.

Number of the strains killed at various time intervals and the amount of bacteria killed were shown in Table 4-7. Extract 1 MIC alone showed bacteriostatic activity (≥1 log CFU/ml were reduced) against 1 strains (5.55%) at 4 hour of growth. ½ MIC of norfloxacin alone showed 90% killing. (≥1 log CFU/ml were reduced) only 1 strain (5.55%) at 4 hour of growth and showed 99% killing. (≥2 log CFU/ml were reduced) against 2 strains (11.11%) at 6 hour of growth and showed bactericidal activity (≥3 log CFU/ml were reduced) against 2 strains (11.11%) at 8 hour of growth.

The combination of extract ½ MIC plus norfloxacin ½ MIC showed 90% killing rated against 1 strain (5.55%) at 2 hour of growth and against 2 strains (11.11%) at 4 hour of growth. Bactericidal activity (≥3 log CFU/ml were reduced) against 1 (5.55%) strain at 4 hour of growth and against 2 strains (11.11%) at 6 hour of growth.

The combination of 1 MIC of extract plus ½ MIC of norfloxacin showed 90% killing rated against 1 strain (5.55%) at 2 hour of growth, against 2 strains (11.11%) , 4 strains (22.22%), 9 strains (50%) and 7 strains (38.88%) at 4, 6, 8, 24 hour of growth, respectively. 99% killing rated against 7 strains (38.88%) at 24 hour of growth and bactericidal activity (≥3 log CFU/ml were reduced) against 1 (5.55%) strain at 2 hour of growth and against 2 strains (11.11%) at 4 hour of growth.

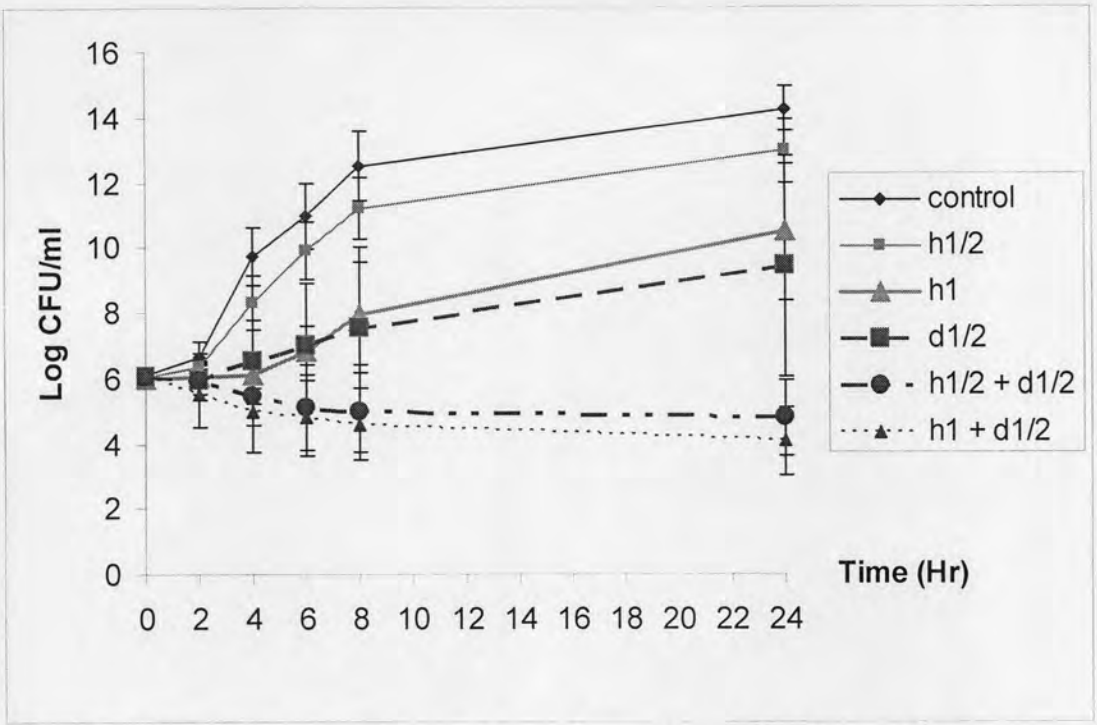

Figure 4- 9 Time kill curves showing the antibacterial actity of the combination of alcoholic extract of *T.citrina* ROXB. plus norfloxacin against 18 strains ESBL-producing *E.coli*

Table 4-6 Mean log change viable counts at various time interval, $AUBKC_{0-24}$ and BA_{24} in 18 isolates of ESBL-producing *E.coli*

condition	Mean(±SD) log change viable cell counts					Mean(±SD) $AUBKC_{0-24}$	Mean(±SD) BA_{24}
	Δ2	Δ4	Δ6	Δ8	Δ24		
control	0.59±0.43	3.61±0.89	4.97±0.99	6.47±1.05	8.16±0.69	286.58±16.00	-
H1/2	0.34±0.37[a]	2.25±0.81[a]	3.91±0.89[a]	5.23±0.92[a]	6.91±0.93[a]	259.88±17.45	27.40±15.51[a]
H1	0.04±0.23[b]	0.08±0.41[b]	0.73±0.98[b]	2.04±1.52[b]	4.17±2.07[b]	202.84±35.49	86.94±31.14[b]
D1/2	-0.05±0.45	0.47±1.28[c,d]	1.04±1.91[c,d]	1.59±2.49[d]	3.39±3.27[c,d]	188.21±54.73	101.17±54.56[c,d]
H1/2+D1/2	-0.11±0.49	-0.61±0.83	-0.92±1.28	-1.02±1.24	-1.21±1.14	121.87±25.61	164.75±30.69
H1+D1/2	-0.49±0.99	-0.98±1.25	-1.21±1.15	-1.40±1.08	-1.93±1.02	110.61±24.40	175.78±30.14

[a] $p< 0.05$ compared to activity of extract ½ MIC plus drug ½ MIC , [b] $p< 0.05$ compared to activity of extract 1 MIC plus drug ½ MIC
[c] $p< 0.05$ compared to activity of extract ½ MIC plus drug ½ MIC , [d] $p< 0.05$ compared to activity of extract 1 MIC plus drug ½ MIC
Δ = Mean log change viable cell counts at 2, 4 , 6 , 8 and 24 hours, respectively
$AUBKC_{0-24}$ = Area under bacterial killing and regrowth curves for 24 hours.
BA_{24} = Bacterolytic area for 24 hours

Table 4-7 Reduction of *E.coli* (18 strains) viable cell counts at various time intervals.

condition	No. of strains to be killed at time point																
	2h			4h			6h			8h				24h			
	-1	-2	-3	-1	-2	-3	-1	-2	-3	-1	-2	-3	R	-1	-2	-3	R
H1/2 MIC	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	14
H 1 MIC	-	-	-	1	-	-	-	-	-	-	-	-	4	-	-	-	16
D1/2	-	-	-	1	-	-	-	2	-	1	-	2	1	1	-	2	12
H1/2+D1/2	1	-	-	2	-	1	-	-	2	1	-	2	-	2	-	2	-
H1+D1/2	1	-	1	2	-	2	4	-	2	9	-	2	-	7	7	2	-

(-1 = 90% of viable reduction versus initial inoculum; -2 = 99% of viable reduction versus initial inoculum; -3 , -4 = 99.9 % of viable reduction versus initial inoculum, R= regrowth)
H = alcoholic extract of *T.citrina* ROXB, D = norflxacin

Antibacterial activities were observed from the time kill study. The comparative activities between the combinations of various MIC levels of both extract and norfloxacin were summarized as followed: (Figure 4-9, Table 4-6 and Table 4-7)

1. Extract ½ MIC alone and the combination of extract ½ MIC plus norfloxacin ½ MIC

The number of bacteria killed by the combination of extract ½ MIC plus norfloxacin ½ MIC [BA_{24} = 164.75 log CFU/ml·h] were significantly higher than the number killed by ½ MIC of extract alone [BA_{24} = 27.40 log CFU/ml·h]($p<0.05$). [Table 4-6]. In addition, the number of strains killed to the level of ≥3 log CFU/ml at 24 hour by ½ MIC of extract alone had no activity against all 18 strains. Bacteria were reduced to the level of ≥3 log CFU/ml by the combination of ½ MIC of extract plus ½ MIC of norfloxacin at 4 hours, only 1 strain(strain no. U16) and the regrowth of 14 strains (strain no. U16, U19, U34, B23, B25, P2, P17, P18, U8, U38, U51, U56, B12, U10) were observed at 24 hours. (Table 4-7)

2. Norfloxacin ½ MIC alone and the combination of extract ½ MIC plus norfloxacin ½ MIC

The number of bacteria killed by the combination of ½ MIC of extract plus ½ MIC of norfloxacin [BA_{24} = 164.75 log CFU/ml·h] were significantly higher than the number killed by ½ MIC of norfloxacin alone [BA_{24} = 101.17 log CFU/ml·h] ($p<0.05$). [Table 4-6] In addition, the number of strains killed to the level of ≥3 log CFU/ml at 24 hour by the combination of ½ MIC of extract plus ½ MIC of norfloxacin (11.76%)

were equal those killed by ½ MIC of norfloxacin alone. But time that cells were reduced to the level of ≥ log CFU/ml by the combination of ½ MIC of extract plus ½ MIC of norfloxacin (1 strain and 2 strains, at 4, 6 hours, respectively) were faster than killing time by ½ MIC of norfloxacin alone (2 strains, at 8 hours). [Table 4-7]

3. Extract 1 MIC alone and the combination of extract 1 MIC plus norfloxacin ½ MIC

The number of bacteria killed by the combination of extract 1 MIC plus norfloxacin ½ MIC [BA_{24}= 175.78 log CFU/ml·h] were significantly higher than the number killed by extract 1 MIC alone [BA_{24}= 86.94 log CFU/ml·h] ($p<0.05$). [Table 4-6]. The number of strains killed to the level of ≥1 log CFU/ml at 4 hour by extract 1 MIC was 1 strain (U16) and the regrowth of 4 strains (strain no.U34, P12, P17, P18) and 16 strains(strain no. U1, U25, U34, B23, B25, P2, P12, P17, P18, U8, U38, U43, U51, U56, B12, U10), were observed at 8, 24 hours, respectively. (Table 4-7)

4. Norfloxacin ½ MIC alone and the combination of extract 1 MIC plus norfloxacin ½ MIC

The number of bacteria killed by the combination of extract 1 MIC plus norfloxacin ½ MIC [BA_{24}= 175.78 log CFU/ml·h] were significantly higher than the number killed by ½ MIC of norfloxacin alone [BA_{24}= 101.17 log CFU/ml·h] ($p<0.05$). [Table 4-6]. In addition, the number of strains killed to the level of ≥3 log CFU/ml at 24 hour by the combination of extract 1 MIC plus norfloxacin ½ MIC (11.76%) were equal those killed by ½ MIC of norfloxacin alone. But time that cells were reduced to the level of ≥ log CFU/ml by the combination of extract 1 MIC plus norfloxacin ½ MIC (1 strain and 2 strains, at 2, 4 hours, respectively) were faster than killing time by norfloxacin ½ MIC alone (2 strains, at 8 hours). [Table 4-7]

5. Combination of extract ½MIC plus norfloxacin ½ MIC and combination of extract 1 MIC plus norfloxacin ½ MIC

The number of bacteria killed by the combination of extract 1 MIC plus norfloxacin ½ MIC [BA_{24}= 175.78 log CFU/ml·h] were significantly higher than the number killed by extract ½MIC plus norfloxacin ½ MIC [BA_{24}= 164.75 log CFU/ml·h] ($p<0.05$) [Table 4-6]. The number of strains killed to the level of ≥3 log CFU/ml at

24 hour by combination of extract ½MIC plus norfloxacin ½ MIC were equal those killed by combination of extract 1 MIC plus norfloxacin ½ MIC (2 strains such as U16, U19). But the number of strains killed to the level of ≥1 log CFU/ml and ≥2 log CFU/ml at 24 hour were difference. Combination of extract 1 MIC plus norfloxacin ½ MIC showed bacteriostatic activity 90% killed in 7 strains (strain no. U25, B25, P2, U8, U51, B12 and U10); 99% killed in 7 strains (strain no. U34, B23, P12, P17, P18, U38 and U56) whereas, combination of extract ½MIC plus norfloxacin ½ MIC showed bacteriostatic activity 90% killed in only 2 strains (strain no. U11, B23). [Table 4-7]