Chapter IV

Results

I. Preliminary screening for antimicrobial activity of some Thai medicinal plants

The antibacterial activities of the crude extracts were shown in Table 11 by inhibition zone diameters and the weight received from the extraction of each plant when macerated 500 g.

Seven plant extracts which inhibited a wide range of test organisms and gave much more clear inhibition distances were selected for further study . There were as followed ;

1. *Putranjiva roxburghii* Wall.
2. *Croton sublyratus* Kurz.
3. *Clerodendrum petasites* Moore
4. *Croton crassifolius* Geisel
5. *Stephania glabra* (Roxb.) Miers.
6. *Terminalia citrina* Roxb. ex. Flem.
7. *Eleutherine palmifolia* (L.) Merr.

Table 11 Antimicrobial activities of plant extracts

Plant	Weight of crude extract (g)	Inhibition zone diameters (mm.)				
		Staphylococcus aureus ATCC 25923	*Pseudomonas aeruginosa* ATCC 27853	*Klebsiella pneumoniae* ATCC 10031	*Streptococcus pyogenes* A 6/49	*Haemophilus influenzae*
Putranjiva roxburghii Wall.	46.7820	18.3	0	0	16.7	0
Croton sublyratus Kurz.	31.5229	18.7	0	0	22.2	19.5
Croton crassifolius Geisel	9.4806	0	14.0	0	14.6	14.2
Dendropthoe pentandra Miq.	21.8785	0	0	0	0	0
Cyperus rotundus Linn.	32.3680	13.6	0	0	0	14.2
Clerodendrum petasites Moore	11.5072	13.1	0	0	15.0	15.9
Lantana camara Linn.	14.3466	0	0	0	14.6	0
Capparis micracantha DC.	32.6857	0	0	0	15.7	0
Stephania glabra (Roxb.) Miers.	36.9622	27.8	0	0	29.3	22.3
Terminalia citrina Roxb.ex.Flem.	25.0909	20.4	15.2	0	18.9	15.6
Eleutherine palmifolia (L) Merr.	23.9365	25.5	0	0	28.4	28.6

II. Antibacterial activities of the plant extracts

Seven residues in I were extratcted with petroleum ether, chloroform, and ethanol. Each extract was weighed and tested for antibacterial activity in order to choose only three extracts for further study. The results were shown in Table 12 and Table 13.

Table 12 Weight of crude extracts

Plant	Weight of extract (g)		
	Petroleum ether	Chloroform	Ethanol
Putranjiva roxburghii Wall.	8.7310	1.0246	24.0584
Croton sublyratus Kurz.	10.9339	4.8496	9.0510
Clerodendrum petasites Moore	2.8209	2.5760	2.6882
Croton crassifolius Geisel	0.7389	0.4402	3.5629
Stephania glabra (Roxb.) Miers.	0.0651	29.6200	5.6004
Terminalia citrina Roxb.ex.Flem.	12.0503	1.2337	6.4722
Eleutherine palmifolia (L.) Merr.	4.9905	2.3856	10.2072

Table 13 Antibacterial activities of plant extracts with different solvents

Plant	Solvent*	Average Inhibition zone diameters (mm.)				
		Staphylococcus aureus ATCC 25923	Pseudomonas aeruginosa ATCC 27853	Klebsiella pneumoniae ATCC 10031	Streptococcus pyogenes A 6/49	Haemophilus influenzae
1. Putranjiva roxburghii Wall.	Pet. ether	0	0	0	0	0
	$CHCl_3$	18.6	0	0	19.2	26.1
	EtOH	20.1	0	0	14.4	16.5
2. Croton sublyratus Kurz.	Pet. ether	0	0	0	0	15.6
	$CHCl_3$	24.9	0	0	22.4	27.5
	EtOH	0	0	0	14.5	14.8
3. Clerodendrum petasites Moore	Pet. ether	0	0	0	0	0
	$CHCl_3$	17.1	0	0	19.3	21.2
	EtOH	0	0	0	0	0
4. Croton crassifolius Geisel	Pet. ether	0	0	0	0	15.0
	$CHCl_3$	17.6	0	0	20.0	20.6
	EtOH	0	0	0	14.1	0

Table 13 (cont.)

Plant	Solvent*	Average Inhibition zone diameters (mm.)				
		Staphylococcus aureus ATCC 25923	Pseudomonas aeruginosa ATCC 27853	Klebsiella pneumoniae ATCC 10031	Streptococcus pyogenes A 6/49	Haemophilus influenzae
5. Stephania glabra (Roxb.) Miers.	Pet. ether	22.8	-**	-**	25.2	17.2
	$CHCl_3$	30.3	0	0	33.4	27.1
	EtOH	15.0	0	0	25.1	15.2
6. Terminalia citrina Roxb.ex.Flem.	Pet. ether	21.3	0	0	15.8	16.4
	$CHCl_3$	18.4	0	0	14.4	0
	EtOH	24.3	19.3	0	16.9	17.7
7. Eleutherine palmifolia (L.) Merr.	Pet. ether	27.6	0	0	27.4	23.1
	$CHCl_3$	19.6	0	0	18.9	16.7
	EtOH	0	0	0	0	0

* Pet. ether = petroleum ether, $CHCl_3$ = chloroform, EtOH = ethanol

** The extract of Stephania glabra (Roxb.) Miers. with petroleun ether wasn't tested against P. aeruginosa ATCC 27853 and K. pneumoniae ATCC 10031 because of the low yield. In addition, the result from Table 6 showed no activity of this plant against these organisms

Eleutherine palmifolia (L.) Merr. extracted with petroleum ether and chloroform and *Stephania glabra* (Roxb.) Miers. extracted with chloroform were selected to determine the antibacterial activity. The results in Table 14 showed that *Eleutherine palmifolia* (L.) Merr. with petroleum ether extraction could inhibit *S.aureus* ATCC 25923 at the concentration of 93.75 µg/disc, *S.pyogenes* A 6/49 at 375 µg/disc, and *H. influenizae* at 187.50 µg/disc.

Table 14 The inhibitory concentrations of *Eleutherine palmifolia* (L.) Merr. and *Stephania glabra* (Roxb.) Miers.

Plant	Test organisms \ Concentration (ug/disc)	Average inhibition zone diameters (mm)							
		46.875	93.75	187.50	375.0	750.0	1250	2500	5000
Eleutherine palmifolia (L.) Merr. with petroleum ether	*Staphylococcus aureus* ATCC 25923	0	15.3	18.3	19.4	20.5	23.2	25.5	26.6
	Streptococcus pyogenes A 6/49	0	0	0	14.8	17.7	20.8	22.5	24.6
	Haemophilus influenzae	0	0	15.6	17.2	18.4	20.4	22.3	24.9
Eleutherine palmifolia (L.) Merr. with chloroform	*Staphylococcus aureus* ATCC 25923	0	0	0	0	0	15.2	18.6	20.3
	Streptococcus pyogenes A 6/49	0	0	0	0	0	16.1	17.9	19.8
	Haemophilus influenzae	0	0	0	0	0	0	16.6	19.5
Stephania glabra (Roxb.) Miers. with chloroform	*Staphylococcus aureus* ATCC 25923	0	0	0	0	0	14.5	17.1	21.7
	Streptococcus pyogenes A 6/49	0	0	0	0	18.1	20.7	23.3	27.7
	Haemophilus influenzae	0	0	0	0	0	14.2	17.6	22.1

III Determination and isolation of antibacterial substances

A. Determination of antibacterial substances by TLC

The *Eleutherine palmifolia* (L.) Merr.petroleum ether extract was developed on pre-coated TLC aluminium sheet and chloroform was used as solvent. Determination by colour shown in UV light(366 nm), there were five main groups of substances (see Figure 2, p 104).

Direct assay was used to find out the location of the antibacterial substances. The inhibition zone was presented only in the third group of TLC sheet, which presented two black spots in UV light (yellow spots in visible light), as shown in Table 15.

Table 15 Location of antibacterial substances of *Eleutherine palmifolia* (L.) Merr. on TLC

Zone diameter (mm) / Test organism	Separated TLC aluminium sheet (group)				
	1	2	3	4	5
Staphylococcus aureus ATCC 25923	0	0	25.2	0	0

B. Isolation of antibacterial substance from Petroleum ether extract of *Eleutherine palmifolia* (L.) Merr. bulb

Column chromatography was used to separated the substance. The solvent system were chloroform : hexane (8:2), cholroform and methanol, respectively. The antibacterial substance was crystallized in hexane yielding yellow needle crystal and was designated as EP_2 (Fig 1, p 103). The development of the crystal on TLC plates with five solvent systems confirmed that the substance obtained was pure.

C. Characterization of Isolated compound

1. Solubility :- EP_2 was soluble in chloroform and acetone; slightly soluble in ethanol; non-soluble in water

2. The Rf values were determined from the chromatoplate

$$Rf = \frac{\text{distance of spot moving from starting point}}{\text{distance of solvent front from starting point}}$$

a.) 0.41 in Silica gel G/ chloroform (Fig 3, p 105)

b.) 0.21 in Silica gel G/chloroform :hexane (8:2) (Fig 4, p 106)

c.) 0.36 in Silica gel G/ chloroform : benzene (8:2) (Fig 5, p 107)

d.) 0.39 in Silica gel G/benzene : acetone (9:1) (Fig 6, p 108)

e.) 0.24 in Silica gel G/petroleum ether : ethyl acetate : chloroform (67:33:10) (Fig 7, p 109)

3. Melting point

166-167° c (yellow needle crystal)

4. Specific rotation

$[\alpha]^{25}_{D}$: -100 (0.1% in chloroform)

5. Ultraviolet absorption spectra

λ_{max} (MeOH) : 236 , 274 (sh.), 293 (sh.) nm.

λ_{max} (EtOH) : 246, 267, 396 nm.

6. Infrared absorption spectrum (KBr disc)

ν_{max} 740, 780, 840, 910, 990, 1050, 1255, 1290, 1450-80 (C-O-C), 1590 (C=C), 1650 (C=O), 1660 (C=O), 2940, 3000 (C-H, benzene ring) cm^{-1}

7. Nuclear magnetic resonance spectrum (300 MHz, TMS as internal reference, $CDCl_3$ as solvent)

δ(ppm) 1.344 (3H,d) CH_3 -1'

1.538 (3H, d) CH_3 -3'

2.233 (1H,ddd) H_{axial} -2'

2.694 (1H, dd) $H_{equatorial}$-2'

3.93-3.99 (1H, m) H-1'

4.007 (3H, s) OCH_3 -(8 or 5)

5.016 (1H, q (broad)) H-3'

7.288 (1H, dd) H-7 or H-6 (proton near-OCH_3)

7.651 (1H, t) H-6 or H-7 (proton in the middle)

7.745 (1H, dd) H-5 or H-8

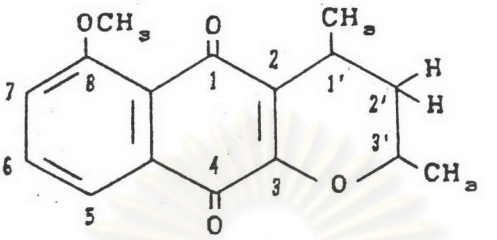
8. Mass spectrum

m/z (% relative intensity)

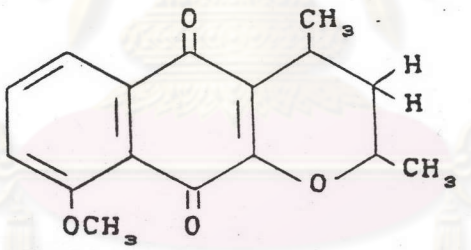
273 (M^+ +1, 18.6), 272 (M^+ , 94.1), 257(100), 244(17.3), 243(50.7), 242(23.1), 229(25.8), 228(11.7), 227 (10), 215(18.4), 214(28.4), 213(18.7), 201(10.3), 135 (11.4), 129(10.2)

The UV, IR, NMR and Mass spectra were concluded for the structre of EP_2 as a naphthoquinone and the molecular formula was assigned as $C_{16}H_{16}O_4$.

The two possible structures of EP_2 (EP_{2A} and EP_{2B}) which were different at the position of $-OCH_3$ substitution (Fig III).



EP_{2A}



EP_{2B}

Figure III Two possible structures of EP_2

Further spectroscopic such as long range decoupling experiment of ^{13}C-NMR, and/or chemical techniques were necessary for the identification of EP_2.

IV Laboratory evaluation of antibacterial activity of EP_2

A. Antimicrobial susceptibility test

50 isolates of each pathogenic organisms, *S. aureus* and *S. pyogenes* obtained from the clinical specimens were tested for antimicrobial susceptibility with 5 various antimicrobial agent discs. The results were shown in Table 16 and Table 17.

B. Determination of Minimal Inhibitory Concentration (MIC) of EP_2

The activity of EP_2 against *S.aureus* and *S.pyogenes* in cumulative percentage of MIC_s (µg/ml) was shown in Table 18. EP_2 inhibited 100% of both *S. aurues* and *S. pyogenes* at the concentration of 40 and 60 µg/ml, respectively.

Figure IV and Figure V showed the relationship between the cumulative percentage of inhibited organisms and log MIC values. By interpolating to the minimal inhibitory concentration of 50 and 90 cumulative percentage inhibited, the MIC_{50} and MIC_{90} were obtained. The overall geometric means and range of the MIC_s of EP_2 against *S. aureus* and *S. pyogenes* were shown in Table 19.

Table 16 Antimicrobial susceptibility patterns against *Staphylococcus aureus*

No.of Specimen	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
1	Cu	S	S	S	R	S
2	Cu	S	S	R	R	R
3	Cu	S	S	S	R	S
4	Cu	S	S	S	R	S
5	Cu	S	S	S	R	R
6	Cu	S	S	S	R	S
7	Cu	S	S	S	R	R
8	Cu	S	S	S	R	R
9	Cu	S	S	S	R	S
10	Cu	S	S	S	R	S
11	Cu	S	S	S	R	R
12	Cu	S	S	S	R	R
13	Cu	S	S	S	R	S
14	Cu	R	R	R	R	R
15	Cu	S	S	S	R	S
16	Cu	S	S	S	R	S
17	Cu	S	S	S	R	S
18	Cu	S	S	S	S	R
19	Cu	S	S	S	R	R
20	Cu	S	S	S	R	R
21	Cu	S	S	S	R	S
22	Cu	S	S	S	R	R
23	Cu	S	S	S	R	S
24	Cu	R	S	R	R	R
25	Cu	S	S	S	I	S

Table 16 (cont.)

No.of Specimen	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
26	Cu	S	S	S	R	S
27	Cu	S	S	S	R	S
28	Cu	S	S	S	S	R
29	Cu	S	S	S	R	S
30	Cu	S	S	S	S	S
31	Cu	S	S	S	R	S
32	Cu	S	S	S	R	S
33	Cu	S	S	S	R	R
34	Cu	S	S	S	R	S
35	Cu	S	S	S	R	S
36	Cu	S	S	S	R	S
37	Cu	S	S	S	R	S
38	Cu	R	R	R	R	R
39	Cu	S	S	S	R	S
40	Cu	S	S	S	R	R
41	Cu	R	S	R	R	R
42	Cu	S	S	R	R	S
43	Cu	S	S	S	R	S
44	Cu	S	S	S	R	S
45	Cu	S	S	S	R	S
46	Cu	S	S	S	R	R
47	Cu	S	S	S	I	S
48	Cu	S	S	S	R	S

Table 16 (cont.)

No.of Specimen	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
49	Cu	S	S	S	R	S
50	Cu	S	S	S	R	R
Total no. of susceptible organisms		46	48	44	3	31
Total no. of tested organisms		50	50	50	50	50
Percent of sesceptible organism		92	96	88	6	62

N.B.

Cu = Chulalongkorn Hospital

S = susceptible; I = intermediate; R = resistant

Table 17 Antimicrobial susceptibility patterns against *Streptococcus pyogenes*

No.of Strains	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
1	Si	S	S	S	S	R
2	Si	S	S	S	S	R
3	Si	S	S	S	S	R
4	Si	S	S	S	S	R
5	Si	S	S	S	S	R
6	Si	S	S	S	S	R
7	Si	S	S	S	S	S
8	Si	S	S	S	S	R
9	Si	S	S	S	S	R
10	Si	S	S	S	S	R
11	Si	S	S	S	S	R
12	Si	S	S	S	S	R
13	Si	S	S	S	S	R
14	Si	S	S	S	S	R
15	Si	S	S	S	S	R
16	Si	S	S	S	S	R
17	Si	S	S	S	S	R
18	Si	S	S	S	S	R
19	Si	S	S	S	S	R
20	Si	S	S	S	S	I
21	Si	S	S	S	S	R
22	Si	S	S	S	S	R
23	Si	S	S	S	S	I
24	Si	S	S	S	S	R
25	Si	S	S	S	S	R

Table 17 (cont.)

No.of Strains	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
26	Si	S	S	S	S	R
27	Si	S	S	S	S	R
28	Cu	S	S	S	S	R
29	Si	S	S	S	S	R
30	Si	S	S	S	S	R
31	Cu	S	S	S	S	R
32	Cu	S	S	S	S	R
33	Cu	S	S	S	S	R
34	Cu	S	S	S	S	R
35	Si	S	S	S	S	R
36	Cu	S	S	S	S	I
37	Cu	S	S	S	S	R
38	Cu	S	S	S	S	S
39	Cu	S	S	S	S	R
40	Cu	S	S	S	S	R
41	Cu	S	S	S	S	R
42	Cu	S	S	S	S	R
43	Cu	S	S	S	S	R
44	Cu	S	S	S	S	R
45	Cu	S	S	S	S	R
46	Cu	S	S	S	S	I
47	Si	S	S	S	S	I
48	Cu	S	S	S	S	R

Table 17 (cont.)

No.of Strains	Hospital	Susceptibility				
		Cephalothin	Clindamycin	Erythromycin	Penicillin	Tetracycline
49	Cu	S	S	S	S	R
50	Cu	S	S	S	S	R
Total no. of susceptible organisms		50	50	50	50	2
Total no. of test organisms		50	50	50	50	50
Percent of sesceptible organism		100	100	100	100	4

N.B. Si = Siriraj Hospital

Cu = Chulalongkorn Hosital

Table 18 Cumulative percentage of *Staphylococcus aureus* and *Streptococcus pyogenes* to MIC (μg/ml) of EP_2

Organism	No. of Strains	Cumulative percentage of isolated strains inhibited at concentrations (μg/ml) of						
		10	20	30	40	50	60	70
Staphylococcus aureus	50	2	32	94	100	100	100	100
Streptococcus pyogenes	50	0	30	66	92	96	100	100

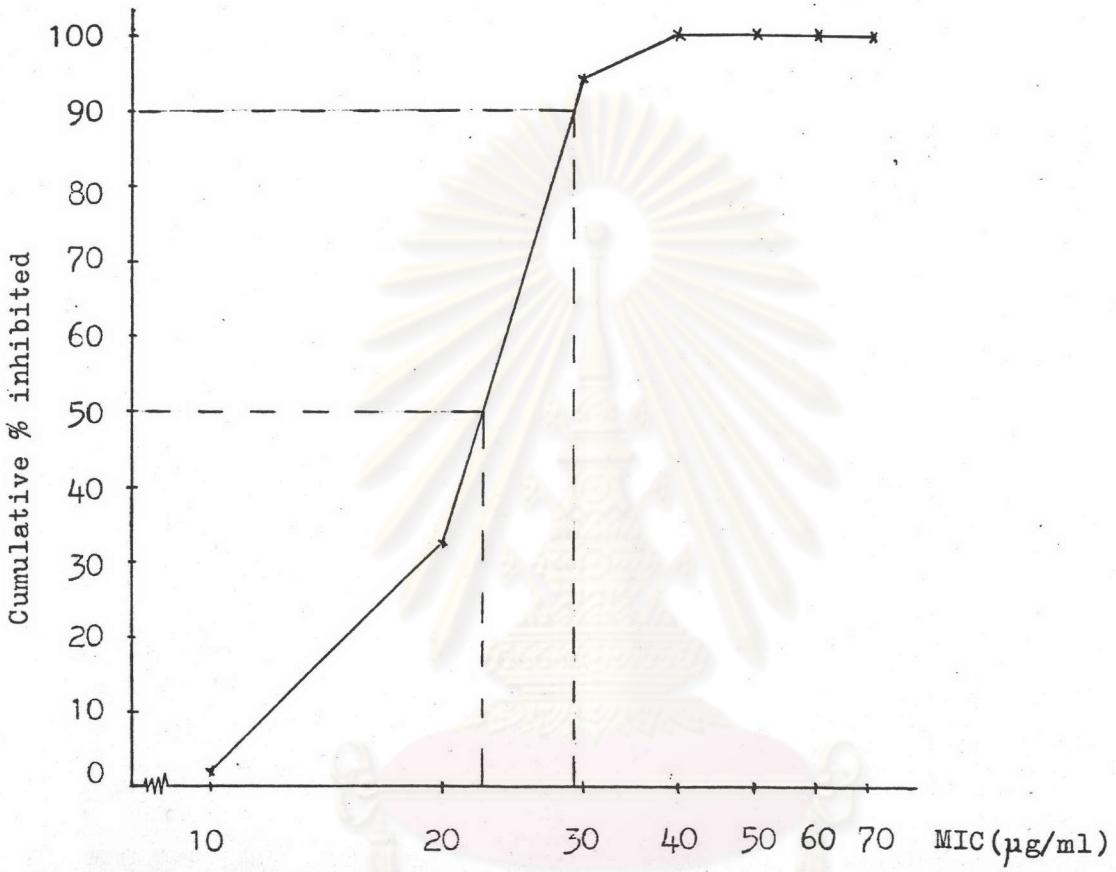


Figure IV The activity of EP_2 against *Staphylococcus aureus*

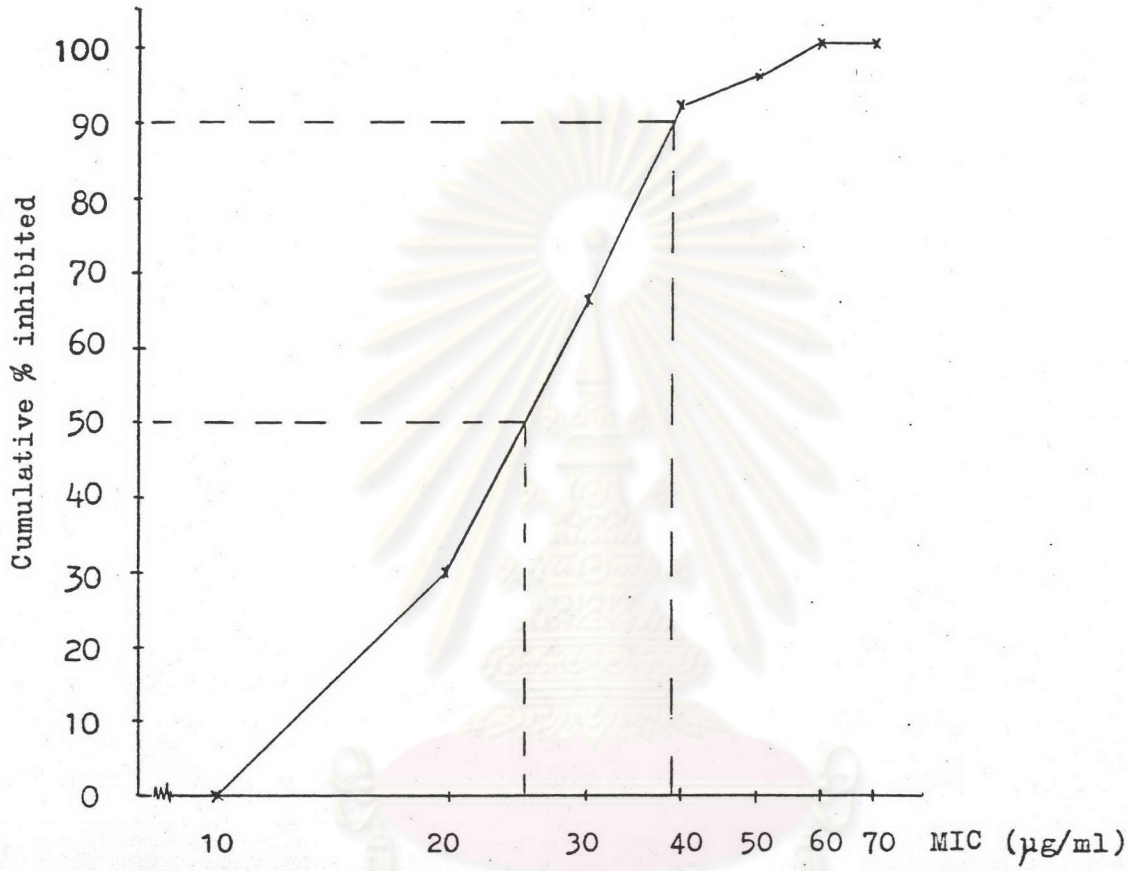


Figure V The activity of EP_2 against *Streptococcus pyogenes*

Table 19 The overall geometric and range of MIC_s of EP_2 against *Staphylococcus aureus* and *Streptococcus pyogones*

Organism	No.of Strains	MIC range (µg/ml)	MIC_{50} (µg/ml)	MIC_{90} (µg/ml)
Staphylococcus aureus	50	10-40	22.5	29.2
Streptococcus pyogenes	50	20-60	25.0	39.0