CHAPTER III

EXPERIMENTS

INSTRUMENTS

1. Infrared Spectrophotometer : Perkin Elmer 1760x, Perkin Elmer 283.

2. Nuclear Magnetic Resonance Spectrophotometer : Bruker FT-NMR (80 MHz), Bruker BZH 200/52 (200 MHz).

3. Mass Spectrometer : Jeol FX 3000 Double Focusing.

4. Melting Point Apparatus : Buchi capillary melting point apparatus (unconnected).

CHEMICALS

4-Aminophenol (BDH).

3-Chloro-4-fluorophenol (Aldrich Chemical co.).

Copper(I)Chloride (Carlo Erba).

Acetic Anhydride (BDH).

Aluminium Chloride (Fluka Chemie AG).

N,N-Dimethylformamide (BDH, Carlo Erba).

Hydroxylamine Hydrochloride (Merk).

Concentrated Sulfuric acid (Baker Analyzed).

All chemicals used were either B.P. or laboratory grade.

Chemical Preparations

1. 4-CHLOROPHENOL (1A)

Dissolved 72 g (0.66 mol) of 4-aminophenol in 170 ml of concentrated hydrochloric acid and 170 ml of water in a 1,000 ml conical flask. The mixture was cooled to 0^{o}c in an ice-salt bath. A solution of 48 g (0.7 mol) of sodium nitrite in 100 ml of water was added to the flask during 10 - 15 minutes, stirred the solution well during diazotization, and keeped the mixture at the temperature of 0 - 5^{o}c .

The cold diazonium chloride solution was slowly poured into the solution of 83.15 g (0.84 mol) Copper(I) chloride in 50 ml of water with continuous shaking. The mixture was allowed to warm to room temperature without external heating but with continuous stiring for 2 - 3 hours. The mixture was then warmed in the water bath to raised the temperature to 60 ^{o}c. This temperature was maintained to complete the decomposition of the double salt until the evolution of nitrogen gas ceased. The mixture was then allowed to cool to room temperature, then the mixture was extracted with chloroform. After chloroform was evaporated off and the

residue was distilled and collected the 4-chlorophenol at 220 ^{o}c. The yield of product was 52 g (61.33 %).

IR (Figure 9)	3360	cm^{-1} (O-H)
(KBr Demountable cell)	1600	cm^{-1} (C=C)
	1240	cm^{-1} (C-OH)
	830	cm^{-1} (para substitution)
^{1}H-NMR (figure 10)	4.67	(s, 1H)
($CDCl_3$)	6.71	(dd, 2H, J=2.3, 6.6 Hz)
	7.10	(dd, 2H, J=2.3, 6.6 Hz)

2. 4-CHLOROPHENYL ACETATE (2A)

Dissolved 25.6 g (0.2 mol) of pure 4-chlorophenol in 128 ml of 10 % sodium hydroxide solution in 500 ml beaker. About 140 g of crushed ice was added, followed by 24 ml (0.25 mol) of acetic anhydride and shaked vigorous for 5 minutes. The mixture was extracted by carbon tetrachloride and then washed the organic layer with 5 % sodium carbonate solution until effervescence ceased. Then, it was separated oily layer and dried with anhydrous sodium sulfate . The product was collected by distillation, 4-chlorophenyl acetate bp. 235 ^{o}c. The yield of product was 32.3 g (95%).

IR (figure 11)	1770	cm^{-1} (C=O)
(KBr Demontable)	1200	cm^{-1} (R-O-C=O)
	850	cm^{-1} (para substitution)

^{1}H-NMR (figure 12)	2.2 (s, 3H)
($CDCl_3$)	6.9 (dd, 2H, J=2.2, 9 Hz)
	7.2 (dd, 2H, J=2.2, 9 Hz)

3. 5-CHLORO-2-HYDROXYACETOPHENONE (3A)

Placed 18.7 g (0.14 mol) of anhydrous aluminium chloride and 40 ml of carbondisulfide in 500 ml two-necked flask with a dropping funnel. The gas absorption trap was attached to the top of condensor. Poured 4-chlorophenyl acetate into the dropping funnel. The suspension was stirred and 4-chlorophenyl acetate was slowly added at such a rate that carbondisulfide boiled vigorously. When all the 4-chlorophenyl acetate had been introduced, the reaction mixture was gently refluxed on the water bath about 2 hours. The reflux condensor was turned downwards and distilled off the solvent. The water bath was replaced with an oil bath and maintained the temperature at 140 ^{o}c for 1 hour. The reaction mixture was allowed to cool and the aluminium chloride complex was decomposed by slowly adding first 150 ml of diluted hydrochloric acid (1:1) and then 250 ml of water . Much heat was evolved and a dark-oil was separated at the bottom, allowed to stand over night to solidify most of 5-chloro-2-hydroxyacetophenone and then filter off and recrystallized from 95% ethanol. The white needle crystal (11 g) of 5-chloro-2-hydroxyacetophenone, mp. 44 - 45 ^{o}c was obtained (51 % yield).

IR (Figure 13)	3400 cm^{-1} (O-H)
(KBr demontable cell)	1660 cm^{-1} (C=O)
^{1}H-NMR (Figure 14)	2.59 (s, 3H, O=C-CH3)
($CDCL_3$)	6.90 (d, 1H, J=9 Hz)
	7.35 (dd, 1H, J= 2.6, 9 Hz)
	7.66 (d, 1H, J=2.5 Hz)
	12.11 (s, 1H, O-H)

4. 6-CHLOROCHROMONE-3-CARBOXALDEHYDE(4A)

The solution of 10 g (0.058 mol) 5-chloro-2-hydroxyacetophenone and 80 ml of N,N-dimethylformamide was stirred in 250 ml round bottom flask. The mixture was then cooled to 0^{o}c in an ice-salt bath. The phosphorous oxychloride 20 ml (0.2 mol) was carefully dropwised during 15 minutes. The reaction mixture was stirred at room temperature for 4 hours and decomposed by adding ice-water . The resulting precipitate was collected by filtration, washed the precipitant with purified water, and recrystallized from acetone to afford 8.2 g (68 %) of pale yellow crystal, (mp. 140 - 141 ^{o}c).

IR (Figure 15)	2860 cm^{-1} (C-H, aldehyde)
(KBr Pellet)	1700 cm^{-1} (C=O, aldehyde)
	1660 cm^{-1} (C=O, pyrone)

^{1}H-NMR (Figure 16)	7.43 (d, 1H, J=9 Hz)
($CDCl_3$)	7.60 (dd, 1H, J=2, 9 Hz)
	8.23 (d, 1H, J=2 Hz)
	8.52 (s, 1H)
	10.35 (s, 1H)

5. 6-CHOLROCHROMONE-3-CARBOALDOXIME (5A)

Dissolved 8 g(0.038 mol) of 6-chlorochromone -3-carboxaldehyde in a warmed absolute ethanol 320 ml and added a solution of 3.12 g(0.045 mol) of hydroxylamine hydrochloride in 8 ml of water. The mixture was stirred at room temperature for 30 minutes. The product was allowed to crystallized . The crystalline aldoxime was filtered off, washed well with purified water and warm absolute ethanol, respectively. The yield of 6-chloro-chromone-3-carboaldoxime 6.37 g (75 %) was obtained, (mp. 192 ^{o}c).

IR (Figure 17)	3200 cm^{-1} (O-H)
(KBr Pellet)	1650 cm^{-1} (C=N,C=O)
^{1}H-NMR (Figure 18)	7.64 (d, 1H, J=9 Hz)
(Acetone-d_6)	7.77 (dd, 1H, J= 2.4, 9 Hz)
	8.07 (d, 1H, J=2.4 Hz)
	8.17 (s, 1H)
	8.53 (s, 1H)
	10.53 (s, 1H)

6. 6-CHLOROCHROMONE-3-CARBONITRILE (6A)

Placed 5 g ($2.2x10^{-2}$ mol) of 6-chlorochromone-3-carboaldoxime and 25 ml of acetic anhydride was placed in 250 ml round bottom flask. The flask was heated cautiously to reflux for 2.5 hours. The reaction mixture was allowed to cool to room temperature. The 10 % sodium carbonate solution was poured into the reaction mixture to neutralize excess acid . The product was solidified, filtered off, washed well with purified water and then recrystallized from acetonitrile to afford 3.1 g (67 %) as a yellow crystal product, (mp.142 - 144^{o}).

IR (Figure 19)	2240 cm^{-1} (C≡N)
(KBr Pellet)	1660 cm^{-1} (C=O)
^{1}H-NMR (Figure 20)	7.73 (d, 1H, J=9 Hz)
(DMSO-d_6)	7.85 (dd, 1H, J= 2.4, 9 Hz)
	7.92 (d, 1H, J=2.4 Hz)
	9.17 (s, 1H)

7. 6-CHLOROCHROMONE-3-CARBOXYLIC ACID (7A)

A solution of 3.0 g ($1.45x10^{-2}$ mol) of 6-chlorochromone-3-carbonitrile and 120 ml of 55 % sulfuric acid was heated at 130^{o}c for one hour. Ice-water (120 ml) was added to the cool solution to precipitate the crude 6-chlorochromone-3-carboxylic acid. The precipitate was recrystallized from ethyl acetate to give 1.9 g (58 %) of product, mp. 192 - 194 ^{o}c.

IR (Figure 21)	2600 - 3150 cm^{-1} (O-H, acid)
(KBr Pellet)	1770 cm^{-1} (C=O, acid)
	1650 cm^{-1} (C=O, pyrone)
	1140 cm^{-1} (O=C-OH)
^1H-NMR (Figure 22)	7.50 (d, 1H, J=9 Hz)
($CDCl_3$)	7.75 (dd, 1H, J=2.5, 9 Hz)
	8.30 (d, 1H, J=2.5 Hz)
	9.00 (s, 1H)

Mass spectral data : 224(4.13%), 180(61.57%), 154(14.26%)
(m/e) 138(9.54%), 126 (7.88%), 110 (3.06%)
(Figure 23) 98(2.80%), 75 (3.81%), 63 (9.29%)

8. 3-CHLORO-4-FLUOROPHENYL ACETATE (1B)

Dissolved 29.31 g (0.2 mol) of 3-chloro-4-fluorophenol in 128 ml of 10 % sodium hydroxide solution in 500 ml beaker. About 140 g of crush ice was added into the mixture followed by the addition of 24 ml (0.25 mol) of acetic anhydride. The mixture was shaked vigorously for 5 minutes. The product was then solidified, filtered this off and washed the precipitate with 5%sodium carbonate solution and purified water, respectively until the filtrate as neutral to litmus. The product was evaporated to dryness under reduced pressure. The yield of 3-chloro-4-fluorophenyl acetate was 35 g (95 %), mp. 38 ^oc .

IR (Figure 24)	1775 cm^{-1} (C=O, ester)
(KBr Pellet)	1190 cm^{-1} (O=C-O-R)
^{1}H-NMR (Figure 25)	2.27 (s, 3H)
($CDCl_3$)	6.9 - 7.3 (m, 3H)

9. 4-CHLORO-5-FLUORO-2-HYDROXYACETOPHENONE (2B)

Placed 15 g (0.08 mol) of dried 3-chloro-4-fluorophenyl acetate was placed in 250 ml round bottom flask and mixed with 32 g (0.24 mol) of anhydrous aluminium chloride . The reaction mixture was heated to 120 ^{o}c for 15 minutes, allowed to cool and then added diluted hydrochloric acid (1:1) and crush ice . The product was solidified, filtered off and washed the precipitate with purified water . The product was recrystallized from 95% ethanol to afford 8.0 g (53.33 %) as a white needle crystal, mp. 65 - 66 ^{o}c.

IR (Figure 26)	3000 cm^{-1} (O-H)
(KBr Pellet)	1640 cm^{-1} (C=O, ketone)
^{1}H-NMR (Figure 27)	2.3 (s, 3H)
($CDCl_3$)	7.01 (d, 1H, J=6 Hz)
	7.42 (d, 1H, J=9 Hz)
	12.00 (s, 1H)

10. 7-CHLORO-6-FLUOROCHROMONE-3-CARBOXALDEHYDE (3B)

A mixture of 4-chloro-5-fluoro-2-hydroxy-

acetophenone 7.5 g (0.04 mol) and 65 ml of N,N-dimethyl formamide were stirred in 250 ml round bottom flask for 5 minutes and then cooled to 0 ^{o}c in an ice-salt bath The phosphorous oxychloride 13.3 ml (0.133 mol) was carefully added dropwisely during 15 minutes. The reaction mixture was stirred at room temperature for 4 hours and decomposed by adding ice-water. The result precipitate was collected by filtration, washed with purified water, and recrystallized from acetone to afford 7.1 g (78 %) as pale yellow crystal, (mp.155 - 156 ^{o}c).

IR (Figure 28)	2880 cm^{-1} (aldehyde proton)
(KBr Pellet)	1710 cm^{-1} (C=O, aldehyde)
	1650 cm^{-1} (C=O, pyrone)
^{1}H-NMR (Figure 29)	7.58 (d, 1H, J=6 Hz)
($CDCl_3$)	7.92 (d, 1H, J=8 Hz)
	8.45 (s, 1H)
	10.28 (s, 1H, aldehyde proton)

11. 7-CHLORO-6-FLUOROCHROMONE-3-CARBOALDOXIME (4B)

Dissolved 6.8 g (0.03 mol) of 7-chloro-6-fluorochromone-3-carboxaldehyde in 300 ml of warm absolute ethanol and added a solution of 2.43 g (0.035 mol) of hydroxylamine hydrochloride in 5 ml of water. The reaction mixture was stirred and allowed to stand at room temperature for 15 minutes. The product was separated as

crystal. Filtered off the crystalline product, washed well with purified water and warm absolute ethanol, respectively . The yield of 7-chloro-6-fluoro-chromone-3-carboaldoxime was 5.8 g (80 %), mp 212 ^{o}c.

IR (Figure 30)	3200 cm^{-1} (O-H)
(KBr Pellet)	1650 cm^{-1} (C=N)
	1620 cm^{-1} (C=O)
^{1}H-NMR (Figure 31)	7.93 (d, 1H, J=9 Hz)
(DMSO-d_6)	8.03 (s, 1H)
	8.18 (d, 1H, J=6 Hz)
	8.70 (s, 1H)
	11.49 (s, 1H)

12. 7-CHLORO-6-FLUOROCHROMONE-3-CARBONITRILE (5B)

Placed 4.8 g(0.02 mol) of 7-chloro-6-fluoro-chromone-3-carboaldoxime and 25 ml of acetic anhydride in 250 ml round bottom flask. The flask was heated cautiously to reflux for 2.5 hours. The mixture was allowed to cooled to room temperature. The 10 % sodium carbonate solution was poured into the flask to neutralize excess acid. The product was solidified, filtered off, washed with purified water and then recrystallized from acetonitrile to afford 3 g (69 %) of 7-chloro-6-fluorochromone-3-carbonitrile, (mp. 138 ^{o}c).

IR (Figure 32)	2240 cm^{-1} (C≡N)
(KBr Pellet)	1660 cm^{-1} (C=O)

^{1}H-NMR (Figure 33)	7.91 (d, 1H, J=9 Hz)
(DMSO-d_6)	8.23 (d, 1H, J=6 Hz)
	9.19 (s, 1H)

13. 7-CHLORO-6-FLUOROCHROMONE-3-CARBOXYLIC ACID (6B)

A solution of 2.8 g ($1.25x10^{-2}$ mol) of 7-chloro-6-fluorochromone-3-carbonitrile and 100 ml of 55% sulfuric acid was heated at 130 ^{o}c for 1.5 hour. Ice-water was added to the mixture to precipitate of crude 7-chloro-6-fluorochromone-3-carboxylic acid. The precipitate was recrystallized from ethyl acetate to give 1.86 g (61 %)of pured product, mp. 155 ^{o}c .

IR (Figure 34)	2700 cm^{-1} (O-H, acid)
(KBr Pellet)	1760 cm^{-1} (C=O, acid)
	1610 cm^{-1} (C=O, pyrone)
^{1}H-NMR (Figure 35)	7.74 (d, 1H, J=6 Hz)
($CDCl_3$)	8.00 (d, 1H, J=8 Hz)
	8.96 (s, 1H)
	12.96 (s, 1H)

Mass spectral data :	242(1.12%), 198(64.11%), 172(11.32%)
(m/e)	156(10.21%), 144 (8.07), 128 (3.11%)
(Figure 36)	116(2.72%), 93 (2.67%), 81 (7.15%)

SCREENING FOR ANTIBACTERIAL ACTIVITY OF 6-CHLOROCHROMONE-3-CARBOXYLIC ACID AND 7-CHLORO-6-FLUOROCHROMONE-3-CARBOXYLIC ACID

The agar diffusion method was used (Bailey and Scott, 1986).

1. Test Medium

Mueller Hinton Agar was used. The ingredient per litre were as followed :-

Beef, Infusion form	300.0	g
Casamino Acids, Technical	17.5	g
Starch	1.5	g
Bacto-Agar	17.0	g

To rehydrate the medium, 38 g was suspended in 100 ml of cold purified water and heated to boiling to dissolve the medium completely. The media was dispensed into the flasks and sterilized in the autoclave for 15 minutes at 15 $lb/inch^2$ pressure ($121^{o}c$) . Excessive heat was avoided during rehydration or sterilization. Final pH was 7.3 ± 0.1 .

2. Preparation of samples

2.1 Each dry product; 6-chlorochromone-3-carboxylic acid and 7-chloro-6-fluorochromone-3-carboxylic acid was dissolved to make the stock concentration of 100

mg/ml by absolute ethanol. The stock concentration was further diluted to make the sample concentration of 15 mg/ml, 30 mg/ml and 60 mg/ml, respectively.

3. Preparation of the inoculum

3.1 Test organisms; three microorganisms were used as followed :

- Staphylococcus aureus ATCC 25923
- Escherichia coli ATCC 25922
- Pseudomonas aeruginosa ATCC 27853

All tested organisms were cultured overnight on Trypticase Soy Agar (TSA) slants at 37 ^{o}c before testing. The ingredients per litre of TSA were as followed :

Pancreatic Digest of Casien USP	15 g
Soy Bean Peptone	5 g
Sodium Chloride	5 g
Bacto-Agar	15 g

40 g of TSA was placed into 1,000 ml distilled water and then boiled until completely dissolved. The medium was sterilized in the autoclave for 15 minutes at 15 lb/inch^2 pressure (121 ^{o}c).

3.2 Each culture was suspended with a small volume of sterile normal saline solution and the inoculum was adjusted to match the turbidity of the standard

Mc Farland solution No.0.5 when comparing the tube against the white background with a contrasting black line (approximately 10^8 CFU/ml).

4. Preparation of test plates

Mueller Hinton Ager (MHA) was melted and allowed to cool to 45 - 50 ^{o}c in a water-bath. Twenty-five millitre of the melted agar medium was dispersed into sterile glass petridish, with internal diameter 9 cm. to yield a uniform dept of about 4 mm. . The agar plate was allowed to harden on a flat level surface. The plates should be incubated at 38 ^{o}c with their lids slightly opened in order to permit the evaporation of surface moisture. Evaporation was usually completed with-in 30 minutes.

5. Inoculation of agar plates

A sterile cotton swab was dipped into each inoculum and the excess inoculum was removed by rotating the swab several times against the inside wall of the tube above the fluid level. The entire surface of the MHA plate was ioculated by streaking with the swab for three times and each time the plate was rotated at 60 degree. This was to ensure an even distribution of inoculum.

6. Application of cups

As soon as possible and not later than 15 minutes after inoculation of the plates, the stainless steel cups (diameters 6 mm.) were applied on the surface of the agar and then filled up the cup with various concentration of sample. The control cup was filled with absolute ethanol.

Cups must be arranged at least 15 mm. from edge of the plate and apart from each other by a distance of 15 - 20 mm. . The plates were left in room temperature for 20 minutes and incubated at 35 - 37 ^{o}c for 18 hours (over night).

7. Result

The zone of inhibition were detected as positive or negative result. Faint growth or tiny colonies near edge of the inhibition zones were ignored if they were presented.