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A novel synthesis of quinoxaline- 6-carabaldehyde and its evaluation as potential antimicrobial agent

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ABSTRACT

Antibiotics medication is prescribed against bacterial infections. Several drug molecules are known to possess antimicrobial activity in addition to their known pharmacological actions like antihistamines, tranquilizers, Antihypertensive, antipsychotics and anti-inflammatory agents. In a quest to explore a newer antimicrobial drug, the present study proposes a novel synthetic process for Quinoxaline- 6-Carabaldehyde, expected to exhibit antimicrobial potency may be due to quinoxaline moiety in the core molecule structure. The structure and purity of the synthesized drug was established by spectral and elemental techniques. It was evaluated for antimicrobial potency in vitro and in vivo. The minimum inhibitory concentration (MIC) of the drug against the bacteria was determined by agar and broth dilution methods in vitro. The antibacterial activity was confirmed by animal experiments. Toxicity and protective efficacy of the drug were tested in vivo. The drug inhibited most of the bacterial isolates tested at 25-100 µg/ml concentration and a few were sensitive even at a lower concentration (10 µg/ml). It was found to be bacteriostatic against "Shigella dysenteriae 7", and bactericidal against "S. aureus NCTC 6571, 8530 and 8531".When administered to swiss white mice the drug protected the animals challenged with 50 MLD of "Salmonella typhimurium NCTC 74". The drug showed inhibitory action against several pathogenic bacteria. It also offered significant protection to mice against the bacterial challenge.

Keywords: Quinoxaline, 4-chloro-2-nitroaniline, 6-chloroquinoxaline, quinoxaline-6-carbaldehyde, bacteriostatic, bactericidal, Pathogenic bacteria.

INTRODUCTION

A wide variety of pharmacological properties has been associated with quinoxaline derivatives.Quinoxaline also called benzopyrazine is a heterocyclic compound containing a ring complex made up of benzene ring and a pyrazine ring has been considered as a wonder nucleus which posses almost all type of biological activities. This includes antidepressant [1], antiviral [2-3], antidiaabetic [4], antimicrobial [5, 6], anticancer [7], anti-inflammatory [8, 9]. Substituted quinoxalines are an important class of benzoheterocycles, which constitute the building blocks of wide range of pharmacologically active compounds. The quinoxaline is described as a bioisoster

of quinoline, naphthalene, benzothiophene and other aromatic rings such as pyridine and pyrazine. Quinoxaline derivatives constitute the basis of many insecticides, fungicides, herbicides, as well as being important in human health and as receptor antagonists. Although rarely described in nature, synthetic quinoxaline moiety is a part of number of antibiotics such as echinomycin, levomycin and actinomycin which are known to be inhibit the growth of Gram-positive bacteria.

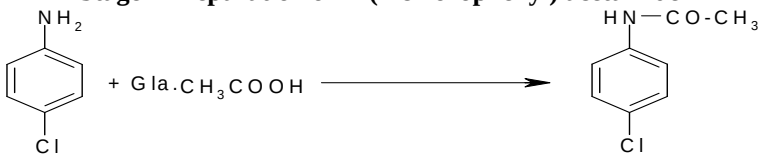
The synthetic process study for preparation of the drug in laboratory was attempted and the developed process was further standardized to achieve consistent quality and yield of the product. The synthesized drug was well characterized using different spectroscopic and elemental techniques to establish structure and confirm purity. In this research Paper we report a six-stage synthesis process for the said drug, its characterization data to establish structure. The research paper further describe results of the study to evaluate antimicrobial potential, toxicity and protective efficacy of the drug using agar and broth dilution methods *in vitro* and *in vivo* respectively.

MATERIALS AND METHODS

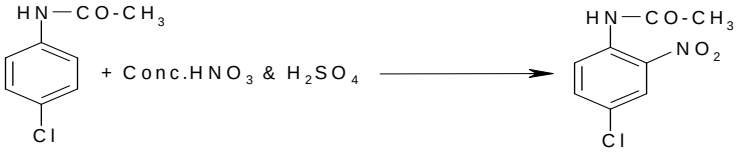
SYNTHETIC:-

Figure 1: Reaction Scheme of proposed synthetic route for target compound.

Stage-I Preparation of N-(4-chlorophenyl) acetamide



Stage-II Preparation of N-(4-chloro-2-nitrophenyl) acetamide



Stage-III Preparation of 4-chloro-2-nitroaniline

Stage-IV Preparation of 4-chlorobenzene-1, 2-diamine

Stage-V Preparation of 6-chloroquinoxaline

Stage-VI Preparation of Quinoxaline- 6-Carabaldehyde

Detailed Synthetic Process:

Stage- Preparation of N-(4-chlorophenyl) acetamide

In Acetic acid (35mol) was added p-Chloro aniline (5 mol) at 20 to 25^{o}C and reaction mixture heat to reflux temperature. A reaction mixture was stirring for 2 hr at reflux temperature. The reaction solution was cool to 65-70^{o}C and diluted with water. Slurry cool to 0 to 5^{o}C and solid filtered from aqueous medium.

Yield 88%yield.

Stage-II Preparation of N-(4-chloro-2-nitrophenyl) acetamide.

To a stirred solution of conc. Nitric acid (12.6mol) was added sulphuric acid (8.3mol) at 0 to 10^{o}C drop-wise and stirred for 15 minutes to form nitrating mixture. Increase temperature to 25^{o}C and add N-(4-chlorophenyl) acetamide lotwise.Maintained yellow reaction mass at 25^{o}C for 1 hour and quenched in cold water slowly. Stirred slurry at 25^{o}C for 2.0 hours and filtered. Wash solid with water. Yield 70%.

Stage-III Preparation of 4-chloro-2-nitroaniline.

To a aqueous solution of 10 % sodium hydroxide (460 ml) add N-(4-chloro-2-nitrophenyl) acetamide. Heat reaction mixture to 70-75^{o}C.Maintained reaction mixture at 70-75^{o}C for 2hour.Cool the reaction mixture to 5-10^{o}C and filtered the solid. After drying at 50-55^{o}C gives 4-chloro-2-nitroaniline with 92% yield

Stage-IV Preparation of 4-chlorobenzene-1, 2-diamine.

To a solution of 4-chloro-2-nitroaniline (1.0 mol) in methanol (5 vol) add Raney Ni (10% by weight) and heat it to 55-60^{o}C in hydrogenater at 10 kg/cm^{2} hydrogen pressure. Maintain reaction mixture at 55-60^{o}C under 10 kg/cm^{2} hydrogen pressure for 2 hr. Filtered reaction mixture at 25-30^{o}C under N_2 atm. through celite. Distill methanol at 45-50^{o}C under vacuum to give 4-chlorobenzene-1, 2-diamine with 94% yield.

Stage-V Preparation of 6-chloroquinoxaline.

In aqueous solution of sodium carbonate (1.15mol in15vol water) add 4-chlorobenzene-1, 2-diamine at 50-55^{o}C followed by lot wise addition of glyoxalbisulfite adduct. Stirred greenish black slurry at 50-55^{o}C for 2 hr. Extract the product in ethyl acetate. Dry organic layer over Na_2SO_4 and distill to get 6-chloroquinoxaline as oil. Yield 75%

Stage-VI Preparation of Quinoxaline- 6-Carabaldehyde.

Under a nitrogen atmosphere, turnings of magnesium were dispersed in THF and Iodine was added. Under a nitrogen atmosphere, a solution of 6-chloroquinoxaline in THF was added

through addition jar initially at room temperature at such a rate gentle reflux (60-65^{o}c) is maintained. Continue the reflux for 1.0h after the addition. Cool reaction mixture to room temperature. Poured the reaction mixture in aqueous solution of NH4Cl and stirred for 15 minute. Extracted the product in ethyl acetate. On recovery of ethyl acetate to get product in oil form. Acetonitrile was added to oil and heat to 60^{o}C and cool to 0-5^{o}C .Filtered the slurry and wash the product with chilled acetonitrile.Product obtained with 82% yield after drying at 50^{o}C.

Pharmacology

Drug:

The synthetic process study and subsequent synthesis of target drug product was carried out in Laboratory at Chemistry Department, Ruparel College, Mumbai 400016.

The synthesized drug substance was well characterized with the help of "IR" using Perkin Elmer FTIR, MS (m/z) using Shimadzu Qp-2010 Mass spectrometer, "H1 NMR" using NMR Bruker, 300 MHz spectrophotometer at "PHL Research Centre", Mumbai. For activity testing drug samples were dissolved in sterile distilled water, and kept at 4^{o}C.

Bacteria:

A total of 414 fully characterized bacterial isolates belonging to 15 genera comprising 100 gram positive and 314 gram negative types were tested. Of these, 46 were "National collection of type culture" (NCTC) and "American type culture collection" (ATCC) strains and preserved in freeze-dried state.

Media:

Peptone water (PW; Oxoid brand, UK), nutrient broth (NB; Oxiod), and Mueller Hinton broth (MHB; Difco, USA) were obtained, and peptone agar (PA), nutrient agar (NA), and Mueller Hinton agar (MHA) were prepared by adding agar to the respective liquid media, according to National Committee for Clinical Laboratory Standards (NCCLS) guidelines [10], and used for determining minimum inhibitory concentration (MIC) of MEB HCl. Streptococcus pyogenes was grown in MHB supplemented with blood. The Anti-microbial testing was carried out at "Department of Toxicology, PHL Research Centre, Mumbai.

Pharmacology Test Protocol for evaluation of antimicrobial potential, toxicity and protective efficacy of the drug.-

Determination of MIC of the drug:

Both broth and agar dilution methods were used to determine the MIC of the drug with respect to different test bacteria [11, 12]. For these methods, Quinoxaline- 6-arabaldehyde was added to each tube or plate at concentrations of 0 (control), 5, 10, 25, 50, 100 and 200 μg/ml. Since one solid agar medium containing the drug could be used for inoculation of a large number of bacteria at a time, this was done at least three times for every test bacterium and the results of agar dilution method only were presented.

Determination of bacteriostatic / bactericidal action of the drug [13]:

Bacterial isolates sensitive to drug molecule were chosen, viz. Shigella dysenteriae 7 and Staphytococcus aureus NTTC 6571, 8530, 8531. The drug was added at a concentration higher than the respective MIC level (50μg/ml) at the logarithmic growth phase of the cultures and colony forming units/ml (cfu/ml) counts were determined at 2 hourly intervals up to 18 hours.

In vivo tests:
Swiss male white mice weighing 18-20g were maintained at the animal house at standard conditions of temperature (21±1°C) and relative humidity (50-60%) with a photoperiod of 14:10h of light-darkness. Water and a dry pellet diet were given ad libitum. The virulence of the test strain Salmonella typhimurium NCTC 74 was exalted by repeated mouse passages and the median lethal dose (MLD

or LD50) of the passaged strain corresponding to $1.85x10^9$cfu/mouse suspended in 0.5 ml NB served as the challenge dose [14] for the test groups of animals.

Toxicity of the drug:
To determine the toxicity of the drug molecule, 10 mice were studied. Of these 5 were injected intraperitoneally 60 μg of the drug, and the rest received 30 μg doze. The animals were kept under observation up to 100 hours.

Protective efficacy of the drug:
Of the two groups of 5 mice each (18-20g) group I was intraperitoneally administrated 30 μg of drug per mouse (0.1 ml from 300 μg/ml solution of drug), and group II was given 60 μg of the drug per mouse (0.1 ml from 600 μg/ml solution of drug). After 3 h, mice in both the groups were challenged with 50 MLD of S. typhimurium 74. A control group of 5 mice were also injected similarly with the same bacterial strain, and 0.1 ml sterile saline instead of drug. The protective capacity of the drug was determined by recording the mortality of the mice in different groups up to 100 hours. In another experiment, 4 groups of three mice each were made. Animals in groups I and III received 60 μg of drug, while animals in groups II and IV received 0.1 ml sterile saline. After 3 h, all groups were given a 50 MLD challenge of S. typhimurium74. Two hours after the challenge, animals of group I and II were sacrificed. The heart blood was collected aseptically; livers and spleens were removed aseptically and homogenized in tissue homogenizers. The cfu/ml homogenate counts of the individual organs were determined separately. The same procedure was applied on groups III and IV, 18h after the challenge. The concentration of drug in mouse blood was assayed by measuring the diameter of the inhibition zones by serum soaked filter paper discs (6mm diameter, 3mm thick, Millipore, USA absorbing 0.03ml volume) on a lawn flooded with 106 bacteria from an 18h broth culture of S. typhimurium 74 on peptone agar. The drug concentrations in serum samples were determined by referring these values to a standard calibration curve prepared with known concentrations of the drugs [15]

RESULTS AND DISCUSSION

Synthetic
Synthetic Process Development Study:
A simple six stage synthetic method was proposed for preparation of Quinoxaline- 6-arabaldehyde. The target drug substance was synthesized in the Laboratory using the proposed method and was obtained in 82.0 % yield. The product isolated was established using different characterization and structure elucidation techniques like physical data, elemental analysis presented in Table1, MS (m/z), 1H NMR and FTIR presented in Table 2.

Table 1: Physical data and Elemental Analysis of the drug and its intermediates

Sr. No.	Compound	Melting Range	Mol Formula	Mol. Wt.	Elemental Analysis Found % and (calculated) %		
					C	H	N
1	N-(4-chlorophenyl) acetamide (Stage I)	175°C	C_8H_8ClNO	169.5	56.62 (56.63)	4.76 (4.76)	8.25 (8.26)
2	N-(4-chloro-2-nitrophenyl) acetamide (Stage II)	103°C	$C_8H_7ClN_2O_3$	214.5	44.71 (44.76)	3.29 (3.29)	13.06 (13.06)
3	4-chloro-2-nitroaniline (Stage III)	113°C	$C_6H_5ClN_2O_2$	172.5	41.75 (41.74)	2.92 (2.92)	16.23 (16.24)
4	4-chlorobenzene-1, 2-diamine (Stage IV)	74°C	$C_6H_7ClN_2$	142.5	50.50 (50.52)	4.96 (4.95)	19.66 (19.65)
5	6-chloroquinoxaline (StageV)	62°C	$C_8H_5ClN_2$	164.5	58.36 (58.36)	3.05 (3.06)	17.05 (17.03)
6	Quinoxaline- 6-arabaldehyde (Stage VI)	131°C	$C_9H_6N_2O$	158.0	68.35 (68.33)	3.85 (3.83)	17.72 (17.72)

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Table 2: Spectral analysis data of drug and Intermediates

Sr. No.	Compound	Spectroscopic Technique	Analysis Results
1	N-(4-chlorophenyl) acetamide (Stage I)	IR (KBr) (cm-1)	3600-3300, 3200-3000, 1700-1640, 540–760 .
		1H NMR Solvent: CDCl3	2.04 (s,3H), 7.23 (s,1H), 7.47 (d,2H), 7.75(d,2H)
		C^{13} NMR Solvent: CDCl3	24.0,120.0,129,133,136,168
		MS (m/z)	169.5
2	N-(4-chloro-2-nitrophenyl) acetamide (Stage II)	IR (KBr) (cm-1)	3369, 1340, 550–770 ,
		1H NMR Solvent: CDCl3	2.0 (s,3H), 7.20 (s,1H), 7.8 (d,1H), 8.0(d,1H), 8.3 (s,1H)
		C^{13} NMR Solvent: CDCl3	24.0,123.9,125.8,129.2,130,135,143.5,169
		MS (m/z)	214.5
3	4-chloro-2-nitroaniline (Stage III)	IR (KBr) (cm-1)	35600-3350, 3330-3100, 1560, 1400, 535–755.
		1H NMR Solvent: CDCl3	6.3 (s, 2H), 6.8 (d,1H), 7.6(d,1H), 8.1 (s,1H)
		C^{13} NMR Solvent: CDCl3	118.6, 123.6, 126.4, 135.7, 138.5, 143.0
		MS (m/z)	172.5
4	4-chlorobenzene-1, 2-diamine (Stage IV)	IR (KBr) (cm-1)	3610-3290 . , 3280-3100 . 530–750 .
		1H NMR Solvent: CDCl3	6.2 (s,4H), 6.3 (d,1H), 6.5(s,1H), 6.6(d1H)
		C^{13} NMR Solvent: CDCl3	117.0,118.5,119.6,125.0,134.5,137.6
		MS (m/z)	142.5
5	6-chloroquinoxaline (StageV)	IR (KBr) (cm-1)	1353 ,1224, 1140 , 1115, 1020 , 955, 914, 870, 834, 530–750.
		1H NMR Solvent: CDCl3	7.1(d,1H), 7.2 (d,1H), 7.4 (s,1H),8.7(d,2H)
		C^{13} NMR Solvent: CDCl3	128.0,130.0,132.0,140.0,142.5,143,145.0 149.0
		MS (m/z)	164.5
6	Quinoxaline- 6-arabaldehyde (Stage VI)	IR (KBr) (cm-1)	1697 ,1353 ,1226, 1142 , 1118, 1024 , 960, 914, 880, 832, 774, 760.
		1H NMR Solvent: CDCl3	7.8 (d,1H), 8.2 (d,1H),8.6 (s,1H), 8.7 (d,2H), 9.8 (s,1H)
		C^{13} NMR Solvent: CDCl3	124.6,130.0,135.8,137.3,140.8,144.6,145.7,147.7, 191.0
		MS (m/z)	158.0

Pharmacology

Pharmacology for evaluation of antimicrobial potential, toxicity and protective efficacy of the drug:

Bacterial inhibitory spectrum:

Of the 414 bacterial isolates tested, were inhibited by the drug at 5-25 µg/ml concentrations presented in Table 3.The staphylococci, vibrios and some enterbacteria like Arizona, Bordetella and Hafnia were sensitive to this drug; 79 of 89 isolates of S. Aureus and 122 of 133 isolates of V. cholerae were inhibited within 100µg/ml concentration. Bacilli and salmonellae were also found to be sensitive to Quinoxaline- 6-arabaldehyde. Resistant isolates mostly belonged to Escherichia coli, Pseudomonas and Klebsiella spp.

Bacteriostatic and bactericidal action:

The MIC of Quinoxaline- 6-arabaldehyde against S. dysenteriae 7 was found to be 25µg/ml. At the logarithmic growth phase of the culture, when the cfu/ml count of the strain was 5.5 x 108, 50 µg/ml of Quinoxaline- 6-arabaldehyde was added. Subsequently, the cfu/ml counts of the

culture were determined after 2, 4, and 6 and at the end of 18h. The counts were 1.0x106, 2.0x104 and 1.5x104/ml respectively. The drug was bacteriostatic on some other Gram negative bacteria like Salmonella typhimurium 74 and Shigella boydii 8. However, Quinoxaline- 6-arabaldehyde proved to be highly bactericidal when tested against S. aureus NCTC 6571, 8530, and 8531.

In vivo toxicity:

No mortality was recorded in the two groups of mice injected with 30 and 60 µg Quinoxaline- 6-arabaldehyde during the observation period of 100h.

In vivo protection:

Of the 5 mice in the control group, 4 (80%) died within 100h of the challenge while 60 and 20 percent mortality was recorded in the two test groups of mice that received 30 and 60 µg of Quinoxaline- 6-arabaldehyde respectively. The difference in the mortality was found to be significant ($p<0.001$) between the drug treated and control groups. Quinoxaline- 6-arabaldehyde significantly reduced the number of viable bacteria in heart blood, liver and spleen of mice in groups I and III both at 2 ($p<0.05$) and 18h ($p<0.01$) after challenge, compared with the control (saline treated) mice as presented in Table 4.

The proposed novel synthetic process for drug substance is well established in the Laboratory to achieve the expected yield and quality of the product. The process was observed to be short, simple, robust, reproducible and with minimal by-product formation. The synthesized drug substance is confirmed by characterization and structure elucidation techniques as described in the write up which supports the validity of proposed synthetic route.

Table 3: *In vitro* activity of the drug on Gram positive and Gram negative bacteria

Sr.No.	Bacteria	Number tested	No. inhibited by Quinoxaline- 6-arabaldehyde µg/ml						
			5	10	25	50	100	200	>200
1	*Bacillus spp.*	7	1	3	2	1	-	-	-
2	*Stephylococusaureu*	89	2	9	32	21	14	4	7
3	*Streptococcus spp.*	4	-		1	-	1	1	1
4	*Escherichia coli*	47	-	1	2	2	2	-	40
Sr.No.	**Bacteria**	**Number tested**	**No. inhibited by Quinoxaline- 6-arabaldehyde µg/ml**						
			5	**10**	**25**	**50**	**100**	**200**	**>200**
6	*Shigella spp.*	48	-	2	8	2	3	13	20
7	*Klebsiella spp.*	8	-	-	-	-	1	1	6
8	*Arizona spp.*	1	-	-	-	1	-	-	-
9	*Providencia spp.*	1	-	-	1	-	-	-	-
10	*Proteus spp.*	4	-	-	-	-	-	1	3
11	*Pseudomonas spp.*	10	-	-	-	-	1	1	8
12	*Pasturella septica*	1	-	-	-	-	-	-	1
13	*Bordetella bronchiseptica*	1	-	-	1	-	-	-	-
14	*Hafnia spp.*	1	-	-	1	-	-	-	-
15	*Vibrio cholerae*	133	5	5	52	29	29	10	1
16	*Vibrio parahemolyticus*	30	1	5	5	6	7	6	-

Table 4: Reduction in cfu/ml of *S.* typhimurium NCTC 74 in organ homogenates of mice treated with drug substance

Time of Sampling (h)	Group	Mouse No.	Drug/Mouse	Cfu/ml counts in		
				Heart blood	Liver	Spleen
2	I	3	Quinoxaline- 6-arabaldehyde 60 µg	1.8×10^3 To $3.2 \times 10^{4*}$	1.4×10^3 To $6.6 \times 10^{4*}$	1.3×10^3 To $2.5 \times 10^{4*}$
2	II	3	Saline (Control)	4.1×10^5 To 6.6×10^6	3.1×10^6 To 9.5×10^6	1.1×10^5 To 8.8×10^6
18	III	3	Quinoxaline- 6-arabaldehyde 60 µg	1.0×10^3 To $4.3 \times 10^{4**}$	4.9×10^3 To $6.7 \times 10^{4**}$	3.1×10^3 To $6.5 \times 10^{5**}$
18	IV	3	Saline (Control)	5.9×10^8 To 8.2×10^9	9.8×10^3 To 7.1×10^9	2.6×10^8 To 7.3×10^9

P < 0.05, **<0.01 compared to control group*

Statistical analysis: The data were analyzed using Student's't'-test and Chi square test.

CONCLUSION

The process can be further scaled up to pilot plant scale to evaluate commercial feasibility.

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REFERENCES

[1]. R.Sarges, H.R. Howard. R.G. Browne, L.A.lebel, P. A. Seymour, B.K. Koe, *J.Med.Chem*.**1990**, 33, 2240.
[2]. G. Campiani, F. Aiello,. M. Fabbrini, E. Morelli, A. Ramunno, S. Armaroli, V. Nacci, A.Garofalo, G.Greco, E. Novellino, G. Maga, S. Sapadari, A. Bergamini, L.Ventura, B. Bongiovanni, M. Capozzi, F. Bolacchi, S. Marini, M. Coletta, G Guiso, S. Caccia, *J.Med.Chem*.**2001**,44,305.
[3]. Z. Zhu, S. Saluja, J. C. Drach, L.B. Townsend, *J.Chin.Chem.Soc*.**1998**, 45, 465.
[4]. M.Y. Chu-Moyer, W.E. Ballinger, D. A. Beebe, R. Berger, J.B. Coutcher, W.W Day, J. Li, B.L. Mylari, P.J. Oates, R.M.Weekly, *J.Med.Chem.***2002**, 45, 511.
[5]. I. M.A Awad, *Indian J.Chem*.**1991**, 30B, 89.
[6].Y.Kurasawa, H. S Kim, *J. Heterocycl.Chem*.**1998**, 35, 1101.
[7]. H.-W Yoo, M.E. Suh, S.W Park, *J.Med.Chem*.**1998**, 41, 4716.
[8]. S.B. Kadin, (Pfizer Inc.) *Ger.Offen*.**1974**, 2,357,186;
Chem.Abstr.**1974**, 81, 37574.
[9]. C. V. Reddy Sastry, K.S Rao, V.S.H. Krishnan, K Rastogi, M.L.Jain, G.K.A.S.S Narayan, *Indian J.Chem*.**1990**,29B,396.
[10].P.A.Villanova, National Committee for Clinical Laboratory standards, Methods for Dilution in antimicrobial Susceptibility Tests for bacteria that grow aerobically. Approved Standard M7-A3, NCCLS, **1993**, 3rd Ed.
[11].E.W. Koneman, S.D. Allen, W.M. Janda, P.C. Schreckenberger. Colormatlas and textbook of diagnostic microbiology, Philadelphia; PA Winn WC, Lippincott; **1997**. 5th Ed 785-856.
[12].Report of working party on antimicrobial sensitivity testing of the British Society for antimicrobial Chemotherapy, a guide to sensitivity testing. *J. Antimicrob Chemother*.**1991**; 27 (Suppl D):1-50.
[13].D.J. Krogstad, R.C. Moellering, Combinations of antibiotics, mechanisms of Interaction against bacteria, Antibiotics in laboratory medicine, London: *Williams and Wilkins*; **1980**.298-341.
[14].L.J. Reed and H.A. Muench,. *Am J Hyg*.**1938**; 27, 493-7.
[15].R.Cruickshank, J.P. Duguid, B.P. Marmion and R.H Swain, Medical microbiology, London: Churchill Livingstone; **1989**.201-208.