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EVALUATION OF INVITRO ANTI-BACTERIAL ACTIVITY OF 5-NITRO 2-THIOPHENE CARBOXALDEHYDE

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ABSTRACT:
Tuberculosis is an infectious disease, caused by the bacterium called *Mycobacterium tuberculosis* which is a slow growing bacteria. It is an airborne communicable disease caused by transmission of aerosolized droplets of *Mycobacterium tuberculosis*, affecting all the organs in the body that are rich in blood and oxygen, the lungs being most commonly affected. The multidrug resistant of bacterial and fungal strains of clinically important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents. The objective of the study is to evaluate anti-tubercular and antimicrobial activity of semisynthetic compound 5- Nitro 2-thiophene carboxaldehyde. The compound is analysed for antimicrobial activity using agar dilution and agar diffusion method & anti-tubercular activity using Microplate Alamar Blue Assay. The results shows that the 5-Nitro 2- thiophene carboxaldehyde has increased bactericidal activity against gram positive bacteria when compared to gram negative bacteria. 5-Nitro 2- thiophene carboxaldehyde at the concentration 7µg/ml seems to be effective against *Staphylococcus aureus*, *Enterococcus*, *E.coli* and *Salmonella typhi* but not against *Pseudomonas aeruginosa*. This compound shows minimum inhibitory concentration at 50µg/ml by preventing the colour change from blue to Pink. Change in colour from blue to pink was scored as growth. So, this compound shows the anti-tubercular activity. It concludes that 5-Nitro 2- thiophene carboxaldehyde shows antibacterial, antifungal and anti-tubercular activity.

KEYWORDS: 5-Nitro 2- thiophene carboxaldehyde, antibacterial, antifungal, anti- tubercular, Microplate Alamar Blue Assay (MABA), agar dilution and agar diffusion.

INTRODUCTION

Tuberculosis is an infectious disease, caused by the bacterium called *Mycobacterium tuberculosis* which is slow growing bacteria. It is an airborne communicable disease caused by transmission of aerosolized droplets of Mycobacterium tuberculosis, affecting all the organs in the body that are rich in blood and oxygen, the lungs being most commonly affected. [1-4 The multidrug resistant of bacterial and fungal strains of clinically

important pathogens fetches the interest of scientist to develop newer broad spectrum antimicrobial agents.[5] The less availability, high cost and greater side effects of new generation antibiotics necessitates looking for the substances from alternative medicines which claimed antimicrobial activity have reported in literature.[6-8] Despite the availability of effective anti- tuberculosis chemotherapy for over 60 years, incidence of tuberculosis continues to persist. The WHO declared tuberculosis as global emergence in 1993.[2] A number of antimicrobial agents exist but the search for new drugs continues, since the target organism evolves into new genetic variant. The chemical efficacy of many existing antibiotic is being threatened by the emergence of multidrug resistance pathogens. The increasing incidence of MDR and XDR tuberculosis worldwide highlights the urgent need to search for anti-tuberculosis drug at increasing probability on finding appropriate drug. The present study was carried out to investigate for its anti-tubercular activity by using H37Rv standard strain and resistant isolates. [9] Organisms develop resistance at a faster rate to the natural antimicrobials because they have pre-exposed to these compounds in nature. Natural antibiotics are often more toxic than synthetic antibiotics. Semisynthetic drug were developed to decrease the toxicity and to increase its effectiveness. Synthetic drugs have an advantage that the bacteria are not exposed to the compound and it was also designed to have even greater effectiveness and less toxicity. 5- Nitro 2- thiophene carboxaldehyde is a synthetic compound which is an intermediate compound to synthesis new derivatives of many novel compounds having excellent antimicrobial, anti-tubercular, anti-inflammatory, anticancer, analgesic, antihistamine, antihistaminic, antiviral, antipsychotic and diuretic activity. In this study, antimicrobial and anti-tubercular activity of 5- Nitro 2- thiophene carboxaldehyde compound has been screened.

Drug Profile

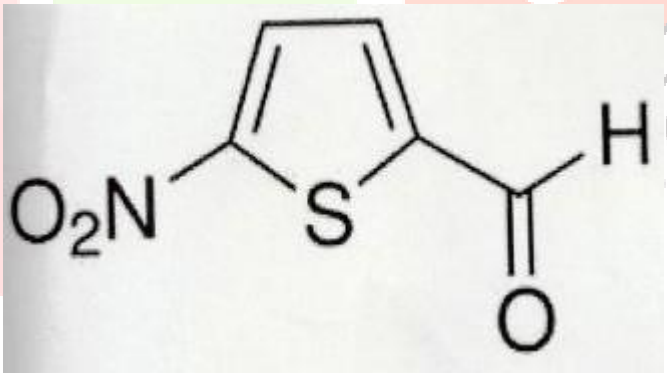

Figure-1.

Molecular formula	: $C_5H_3NO_3S$
Molecular weight	: 157.15 gm/mol
Colour	: Yellow to brown
Form	: Solid (crystals, fibres, crystalline powder)
Melting point	: 75-77º C
Purity	: >97.5%
Solubility10mg/ml (1%), Acetone	: Clear
Solubility colour	: Colourless to yellow colour Incompatible material : Strong oxidizing agent, strong base.

Description

5- Nitro 2- thiophene carboxaldehyde is an aromatic heterocyclic compound consisting of four carbon atoms and one sulphur atom in a five membered ring with nitro group at the 5th position. Thiophene was discovered by Victor Meyer in 1883 as a contaminant in benzene.[10] Thiophene and its derivatives occur in petroleum, sometimes in concentration up to 1-3%. The thiophenic content of liquids from oil and coal is removed via the Hydro- Desulphurization (HDS) process.[11]

MATERIALS AND METHODS

The Compound 302295 (Compound name) was purchased from Sigma-Aldrich Pvt Ltd, Bangalore, Karnataka.

Antibacterial activity

5-Nitro 2- thiophene carboxaldehyde was subjected to antibacterial studies against Gram positive and Gram negative organisms. The organisms used were *Staphylococcus aureus, Enterococcus, E.coli, Pseudomonas aeruginosa, Salmonella typhi*. These organisms were maintained on nutrient agar slopes at 4^{o} C in the Institute of Microbiology, Madras medical college, Chennai and the organisms were confirmed by biochemical tests.

Medium: Muller Hinton Agar Media were obtained from Hi-media Laboratory, Mumbai - 400865, India.

Preparation of the Bacterial Suspension for Inoculation[12]

Few colonies of the pathogenic strains were picked and inoculated into 4ml of peptone water. These tubes were incubated for 2-5 hrs to produce a bacterial suspension. The suspension was then diluted if necessary with the saline solution to a density, visually equivalent to that of Standard which was prepared by adding 0.5ml of 1% barium chloride to 99.5ml of 1% of sulphuric acid. This suspension was then used for seeding.

Preparation of 5-Nitro 2- thiophene carboxaldehyde for microbial activity testing

The compound dilution were prepared by dissolving the 5-Nitro 2- thiophene carboxaldehyde in DMSO (Dimethyl Sulfoxide) and make up the volume using distilled water. Similarly, Standard drugs (Ciprofloxacin, Gentamicin, and Amikacin) were diluted.

Table-1.

MPOUND NAME	CONCENTRATION	COMPOUND (ml)	AGAR MEDIUM (ml)	TOTAL MEDIUM VOLUME(ml)
5-Nitro 2-thiophene carboxaldehyde	1 µg/ml	1.2	13.8	15
	2µg/ml	1.2	13.8	15
	3µg/ml	1.2	13.8	15
	4µg/ml	1.2	13.8	15
	5µg/ml	1.2	13.8	15
	6µg/ml	1.2	13.8	15
	7µg/ml	1.2	13.8	15
	8µg/ml	1.2	13.8	15
	9µg/ml	1.2	13.8	15
	10µg/ml	1.2	13.8	15

Preparation of agar plates

The plates were prepared using Mueller Hinton Agar Medium and different compounds (5- Nitro 2- thiophene carboxaldehyde, Ciprofloxacin, Gentamicin, Amikacin) of various dilutions allowed to solidify and dry. Then, a loopful of different bacterial cultures was inoculated at the labelled spots. All the plates were then incubated at 37° C for 24 hrs and the results were recorded.

Inoculum preparation: The inoculum was standardized at 1.5x 10^8 CFU/ml, by comparing with turbidity standard (0.5 Mac Farland tube).

Swab preparation: A cotton swabs on wooden applicator sticks was prepared. They were sterilized in tins, culture tubes or on paper, either in the autoclave or by dry heat.

ANTIFUNGAL ACTIVITY

5- Nitro 2- thiophene carboxaldehyde was subjected to antifungal activity. The fungal strains used are *Candidia albicans* and *Aspergillus flavus.* These organisms are maintained on Sabouraud's dextrose agar media at 4°C. The dilutions were prepared by dissolving the 5- Nitro 2- thiophene carboxaldehyde in DMSO and dilutions done by using distilled water.

Table-2.

COMPOUND NAME	CONCENTRATION (µg/ml)	COMPOUND(ml)	SABOURAUD'S MEDIUM (ml)	TOTAL MEDIUM VOLUME (ml)
5- Nitro 2-thiophene carboxaldehyde	1µg/ml	1.2	3.8	5
	3µg/ml	1.2	3.8	5
	5µg/ml	1.2	3.8	5
	7µg/ml	1.2	3.8	5
	9µg/ml	1.2	3.8	5
	10µg/ml	1.2	3.8	5

Preparation of Sabouraud's Medium

The plates were prepared using Sabouraud's dextrose agar medium and 5- Nitro 2- thiophene carboxaldehyde of various dilutions allowed to saponify and dry. Then, a loopful of different bacterial cultures was inoculated at the labelled spots. All the plates were then incubated at 37° C (*Candida albicans* for 24hrs and *Aspergillus flavus* for 48hrs) and results were recorded.

Determination of Minimum inhibitory concentration by agar dilution method

The prepared and sterilized medium (15ml for bacterial strain and 5ml for fungal strains) was poured in sterilized petridish in which different concentrations of drug test and standard drugs were added. Then, it is allowed to solidify on a plane table. Mark the plate with a marker to differentiate various organisms in the same plate and then a loopful of different bacterial cultures was inoculated at the corresponding labelled spots. Bacterial strains used are *Staphylococcus aureus, Enterococcus, E.coli, Pseudomonas aeruginosa, Salmonella typhi;* fungal strains are *Candida albicans* and *Aspergillus flavus*. The strains inoculated in all the plates were incubated at 37°C for 24 hrs except *Aspergillus flavus* because which requires 48hrs incubation. Then, the result was recorded.

Zone Of Inhibition for Various Bacterial Strains by using Agar Diffusion Method

All of the compounds (5- Nitro 2- thiophene carboxaldehyde, Ciprofloxacin, Gentamicin, Amikacin) were dissolved in corresponding solvent and diluted with water at the various concentrations (8, 16, 32, 64µg/ml) for testing antibacterial activity. The compound diffused into the medium produces a concentration gradient. After the incubation period, the diameter of the zone of inhibition was measured in millimetre. The plates were inoculated by dipping a sterile swab into inoculums. Excess inoculum was removed by pressing and rotating the swab firmly against the side of the tube, above the level of the liquid. The swab was streaked all over the surface of the medium three times, rotating the plate through an angle of 60° after each application. Finally, the swab was passed round the edge of the agar surface. The inoculation was dried for a few minutes, at room temperature with the lid closed. Ditch the bore in agar plate, add drug solution in bore. Within 30 minutes of preparation of bacterial suspension, the plates were placed in an incubator at 37° C. After 24 hrs, the diameter of inhibitory zone was measured and recorded in millimetre. The measurements were taken with a ruler, from the bottom of the plate without opening the lid.

ANTI-TUBERCULAR METHOD

Minimum inhibitory concentration of the Mycobacterium tuberculosis bacterium (H37Rv) is determined by Microplate Alamar Blue Assay method (MABA). The sputum specimen was collected and several biochemical tests have been described for the identification of mycobacterial species:[13,14]

i. **NIACIN TEST:** If niacin has been extracted from the culture, a yellow colour will appear in the extract within a few minutes.

ii. **ARYLSULPHATASE TEST:** A pink colour indicates a positive reaction.
Other test includes neutral red test, catalase peroxidase test, amidase test, nitrate reduction test.

Medium used: Lowenstein Jensen medium (for maintenance of Mycobacterium tuberculosis culture) and 7H9 broth (for growth of Mycobacterium tuberculosis bacterium).

INOCULALTION AND INCUBATION PROCEDURES

Inoculation Procedures: Two slopes per specimen are inoculated each with one 5 mm loopful of the centrifuged sediment, distributed over the surface. 100µl of 0.5 x 10^6ml of the Mycobacterium tuberculosis (H37Rv) was cultured in 7H9 medium in the presence of compound. Bottle caps should be tightened to minimize evaporation and drying of media. Care should be taken to avoid using red hot loop and loop should be cooled before inoculation.

INCUBATION PROCEDURE

All cultures should be incubated at 35-37°C until growth is observed or discarded as negative after eight weeks. Contaminated slopes are also discarded. Middle brook 7H9 Broth base with added enrichment is recommended for cultivation and sensitivity testing of Mycobacterium tuberculosis.

Directions: Suspend 2.35 gms in 450 ml distilled water, add either 2ml glycerol or 0.5 gm polysorbate 80. Heat if necessary to dissolve the medium completely and sterilize it by autoclaving at 15 lbs pressure (121° C) for 10 minutes. Cool to 45° C or below and aseptically add 1 vial Middle brook ADC Growth Supplement (FD019).

Procedure

200 µl of sterile deionized water was added to all outer perimeter wells of sterile 96 wells plate to minimized evaporation of medium in the test wells during incubation. To 96 well plate, add 100µl of the Middle brook 7H9 broth and serial dilution of 5- Nitro 2- thiophene carboxaldehyde and standard Pyrazinamide were added directly on plate. The control plate is made with 7H9 broth and bacterium only. The final drug concentrations tested were 100 to 0.8µg/ml. Plates were covered and sealed with parafilm and incubated at 37° C for 5 days. After this time, 25µl of freshly prepared 1:1 mixture of Alamar blue reagent and 10% Tween 80 was added to the plate and incubated for 24 hrs. A blue colour in the well was interpreted as no bacterial growth, and pink colour was scored as growth. The MIC was defined as lowest drug concentration which prevented the colour change from blue to pink.[15]

RESULTS

I. MINIMUM INHIBITORY CONCENTRATION

a. FOR BACTERIAL STRAINS

Table 3: 5- Nitro 2- thiophene carboxaldehyde.

Drug concentration (µg/ml)	Organism				
	Gram positive		Gram negative		
	Staphylococcus aureus	*Enterococcus*	*E.coli*	*Pseudomonas aeruginosa*	*Salmonella typhi*
1µg/ml	+	+	+	+	+
2µg /ml	+	+	+	+	+
3µg /ml	+	+	+	+	+
4µg/ml	-	+	+	+	+
5µg/ml	_	+	+	+	+
6µg/ml	-	-	+	+	+
7µg/ml	-	-	-	+	-
8µg/ml	-	-	-		-

(+) : bacterial growth (-) : inhibition of bacterial growth

Table 4: CIPROFLOXACIN.

Drug Concentration (µg/ml)	Organism				
	Gram positive		Gram negative		
	hylococcus aureus	*Enterococcus*	*E.coli*	*seudomonas aeruginosa*	*monella typhi*
1µg/ml	+	+	+	+	+
2µg/ml	+	+	+	+	+
4µg/ml	-	+	-	+	-
8µg/ml	-	+	-	-	-
16µg/ml	-	-	-	-	-

(+) : bacterial growth ; (-): inhibition of bacterial growth

Table 5: GENTAMICIN.

Drug Concentration µg/ml	Organism				
	Gram positive		Gram negative		
	hylococcus aureus	*Enterococcus*	*E.coli*	*seudomonas aeruginosa*	*monella typhi*
1µg/ml	+	+	+	+	+
2µg/ml	-	+	+	+	-
4µg/ml	-	+	+	-	-
8µg/ml	-	-	-	-	-
16µg/ml	-	-	-	-	-
32 µg/ml	-	-	-	-	-

(+): bacterial growth (-): inhibition of bacterial growth.

Table 6: AMIKACIN.

Drug Concentration in µg/ml	Organism				
	Gram positive		Gram negative		
	hylococcus aureus	**Enterococcus**	**E. coli**	**seudomonas aeruginosa**	**monella typhi**
4µg/ml	+	+	+	+	+
8µg/ml	-	+	+	+	+
16µg/ml	-	+	-	-	-
32µg/ml	-	-	-	-	-
64µg/ml	-	-	-	-	-

(+): bacterial growth (-): inhibition of bacterial growth

b. FOR FUNGAL STRAINS

Table 7: 5-NITRO 2-THIOPHENE CARBOXALDEHYDE.

DRUG CONCENTRATION	Organism	
	Candida albicans	*Aspergillus flavus*
1µg/ml	+	+
3µg/ml	+	+
5µg/ml	+	+
7µg/ml	+	+
9µg/ml	-	+
10µg/ml	-	-

(+): fungal growth (-) : inhibition of fungal growth

II. ZONE OF INHIBITION BY AGAR WELL DIFFUSION METHOD Table-8.

DOSE IN µg/ml	DRUG NAME	Organisms (Zone of inhibition in millimetre)				
		S. aureus	*Enterococcus*	*E.coli*	*P. aeruginosa*	*S. typhi*
8µg/ml	5-nitro 2-thiophene carboxaldehyde	13	14	11	12	10
	Ciprofloxacin	20	18	18	22	12
	Gentamicin	15	16	10	11	11
	Amikacin	14	16	10	10	10

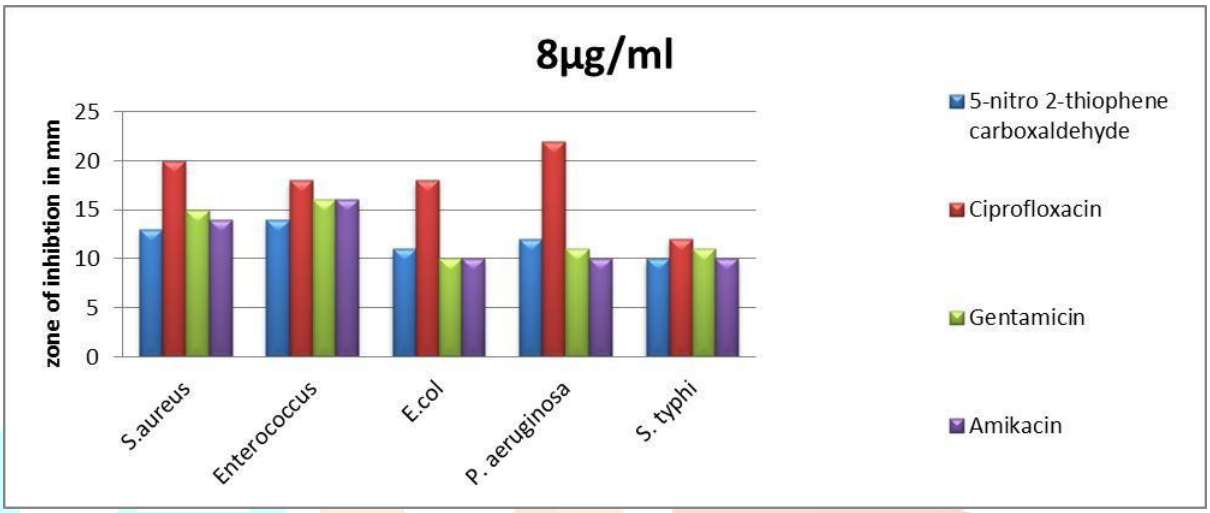

Figure-2.

Table-9.

DOSE (µg/ml)	DRUG NAME	Zone of inhibition in millimetre(diameter)				
		S. aureus	Enterococcus	E.coli	P.aeruginosa	S. typhi
16µg/ml	5-nitro 2-thiophene carboxaldehyde	20	18	18	30	17
	Ciprofloxacin	22	21	20	28	25
	Gentamicin	18	16	15	15	16
	Amikacin	19	17	16	15	17

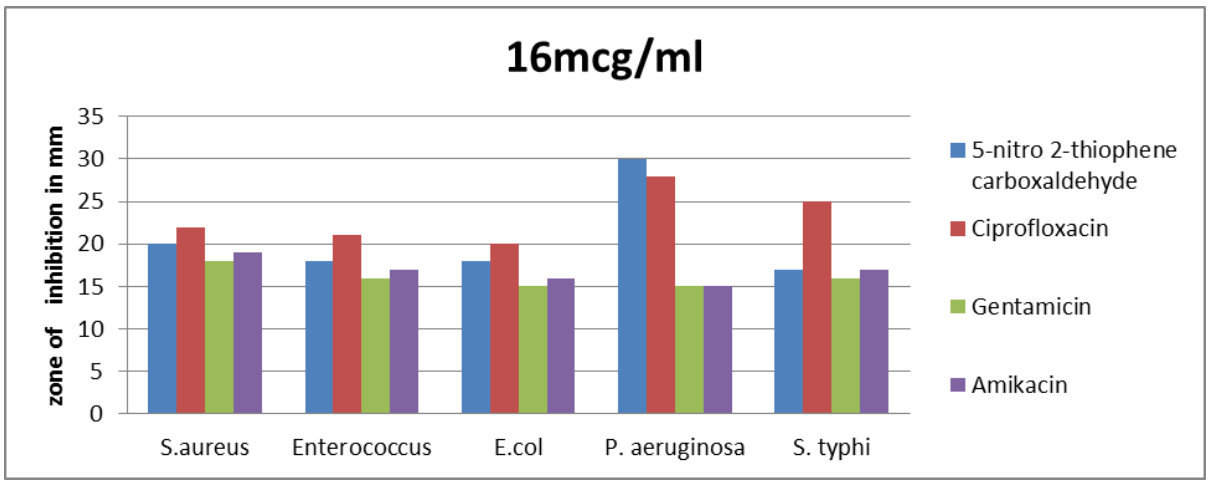

Figure-3.

Table-10.

DOSE IN µg/ml	DRUG NAME	Diameter Of Zone of inhibition in mm				
		S. aureus	*Enterococcus*	*E.coli*	*P.eruginosa*	*S.typhi*
32µg/ml	5-nitro 2-thiophene carboxaldehyde	24	19	19	38	30
	Ciprofloxacin	23	22	21	32	26
	Gentamicin	20	16	16	20	16
	Amikacin	20	17	20	28	17

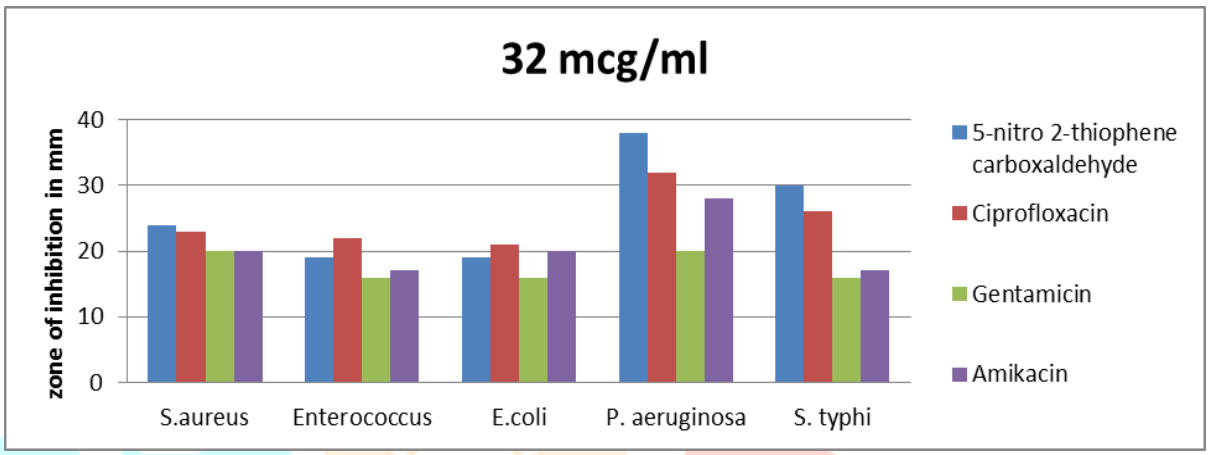

Figure-4.

Table-11.

Dose In µg/ml	DRUG NAME	Diameter Of Zone of inhibition in mm				
		S.aureus	*Enterococcus*	*E.coli*	*P.aeruginosa*	*S. typhi*
64µg/ml	5-nitro 2-thiophene carboxaldehyde	25	24	26	40	43
	Ciprofloxacin	25	25	22	35	32
	Gentamicin	20	18	21	20	20
	Amikacin	22	19	25	28	25

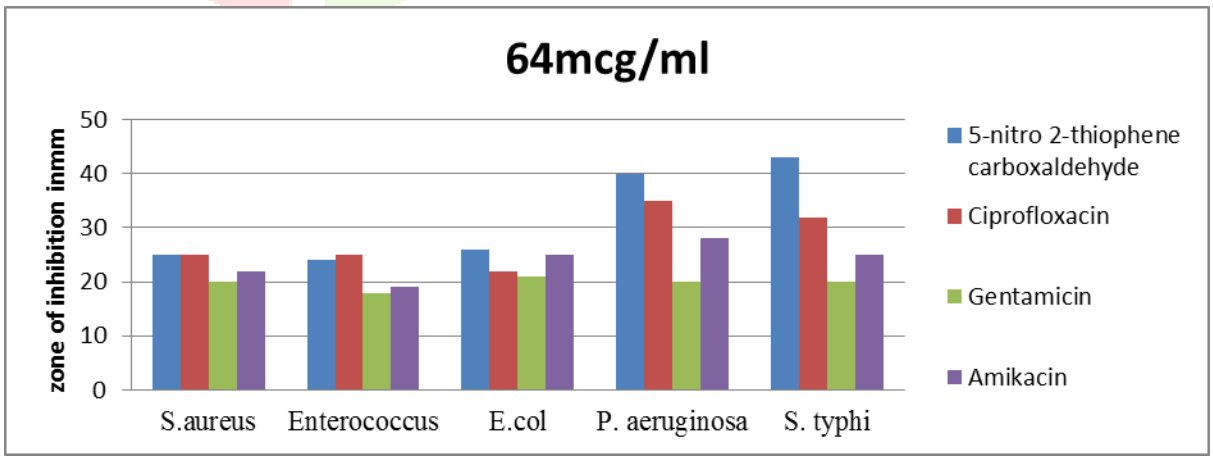

Figure-5.

ANTI-TUBERCULAR ACTIVITY

Table-12.

SAMPLE NAME		100 µg/ml	50 µg/ml	25 µg /ml	12.5 µg /ml	6.25 µg/ml`	3.12 µg /ml	1.6 µg/ml	0.8 µg/ml
5-Nitro 2- thiophene carboxaldehyde		S	S	R	R	R	R	R	R

S- SENSITIVE **R- RESISTANCE**

Strain used: Mycobacterium tuberculosis (H37RV)

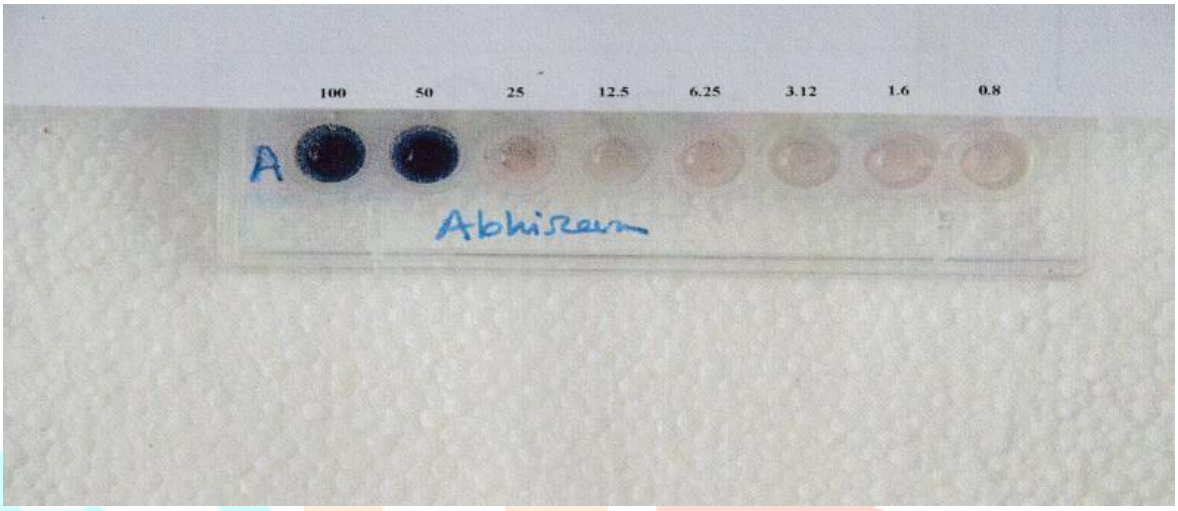

Figure 6: Minimum inhibitory concentration of 5-nitro 2-thiophene carboxaldehyde.

DISCUSSION:

ANTIMICROBIAL ACTIVITY FOR BACTERIAL STRAINS

The results shows that the 5-Nitro 2- thiophene carboxaldehyde has increased bactericidal activity against gram positive bacteria when compared to gram negative bacteria. 5-Nitro 2- thiophene carboxaldehyde at the concentration 7µg/ml seems to be effective against *Staphylococcus aureus, Enterococcus, E.coli* and *Salmonella typhi*, but not against *Pseudomonas aeruginosa*. 8µg/ml is the critical break point minimum inhibitory concentration for the 5-Nitro 2- thiophene carboxaldehyde compound. At this concentration, all the tested pathogens were inhibited.

STANDARD DRUGS

Ciprofloxacin has better gram negative activity than gram positive organisms. At the concentration of 8µg/ml, all the tested gram negative bacteria and *Staphylococcus aureus* were inhibited. 16µg/ml is the critical break point of minimum inhibitory concentration for the Ciprofloxacin standard drug. At this concentration all the drug test pathogens were inhibited; Gentamicin seems to have good gram positive and gram negative activity even at 4µg/ml. 8µg/ml is the critical break point of MIC; Amikacin has the best broad spectrum activity at 16µg/ml and its critical break point of MIC is 32µg/ml.

FOR FUNGAL STRAINS

5-Nitro 2- thiophene carboxaldehyde at the concentration of 9µg/ml completely inhibited the *Candida albicans* and *Aspergillus* flavus was moderately inhibited. 10 µg/ ml is the critical breakpoint of MIC for the 5-Nitro 2- thiophene carboxaldehyde compound. At this concentration, both yeast like fungi and moulds were

completely inhibited.

ZONE OF INHIBITION: Preliminary antibacterial activity was carried out for 5-Nitro 2- thiophene carboxaldehyde compound using Agar well diffusion method against bacterial strains like *Staphylococcus aureus, Enterococcus, E.coli, Pseudomonas aeruginosa, Salmonella typhi* by comparing with the Standards like Ciprofloxacin, Gentamicin and Amikacin. The compound shows good antibacterial activity on Agar diffusion methods by measuring the diameter of the inhibitory zone. The 5-Nitro 2- thiophene carboxaldehyde shows antibacterial activity at concentration dependent manner. The diameter of zone of inhibition was more in *Pseudomonas aeruginosa and Salmonella typhi* at high concentrations than other organisms like *Staphylococcus aureus, Enterococcus and E.coli*. The increasing order of zone of inhibition based on the diameter of the organisms: *P. aeruginosa > S.typhi> Enterococcus>E. coli*. When compared to standards, the diameter of inhibitory zone at all increasing concentration is relatively equal to that of the Ciprofloxacin. So this drug has significant antibacterial activity. 5-Nitro 2- thiophene carboxaldehyde showed best antimicrobial activity at very low concentration (8µg/ml). Its potency was greater than that of standard. Under the standard laboratory conditions at $1.5x10^8$ CFU (colony forming unit/ml), it has been found that 8 µg/ml of 5-Nitro 2-thiophene carboxaldehyde seems to be effective against *S. aureus, Enterococcus, E.coli, P. aeruginosa and S. typhi*. But under the practical condition, 5-Nitro 2- thiophene carboxaldehyde would be highly effective against most of the commonly isolated pathogens.

Anti-Tubercular Activity

Invitro anti-tubercular activity of 5-Nitro 2- thiophene carboxaldehyde was determined by Microplate Alamar Blue Assay (MABA) method. After 24hrs of incubation at 37°C, the inhibition of growth of M.tuberculosis in the compound containing well were recorded from the concentration of 100µg/ml to 0.8µg/ml. This compound 5-Nitro 2- thiophene carboxaldehyde shows MIC at 50µg/ml by preventing the colour change from blue to Pink. Change in colour from blue to pink was scored as growth. So this compound shows the anti- tubercular activity.

CONCLUSION

Multiple drug resistance has developed due to the indiscriminate use of commercial antimicrobial drugs commonly used in the treatment of infectious diseases. In addition to this problem, antibiotics are sometimes associated with adverse effect on the host including sensitivity, immune supervision and allergic reaction. This situation forced scientist to search for new antimicrobial substances. Given the alarming incidence of antibiotic resistances in anti-bacterial of medical importance, there is a constant need for new and effective therapeutic agents. The new compound, 5-Nitro 2- thiophene carboxaldehyde shows significant antibacterial, antifungal and anti-tubercular activity.

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