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ANTIMICROBIAL ACTIVITY OF ORGANIC NLO CRYSTALS USING THIOSEMICARBAZONE DERIVATIVE

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ABSTRACT

Thiosemiccarbozones are having enormous medicine applications due to the presence of hetero atom nitrogen and sulfur in its molecular structure. The carbonyl compounds are also having the largest clinical applications to cure many diseases. We merged these two classes of organic compounds and their substitution by solution growth techniques of crystal growth tested as antibiotic against few fastidious and non fastidious gram +ve and gram –ve organisms. Ortho substituted para substituted and Meta substituted benzaldehydes were merged with thiosemicarbazone and prepared. Thiosemicarbazone of (2-chlorophenyl) methylideneamino thiourea, when compared with linear optical material, these NLO have high medicinal application since it is the photo dynamic therapeutically more active, proved by the early researches mentioned in the experimental part. These one compounds were tested against. Streptococcus Aureus, the following experimental method was adopted find out the antimicrobial activity. MTT assay by colorimetric method, Cell viability percentage calculation method, Half inhibitory maximum (IC50) by calculation method. Agar disc diffusion method. Inhibition zone width by agar disc diffusion method, EUCAST and NCCLS database analysis to compare the MIC and inhibition zone width of existing antibiotic against the above said one organism, Graphpad prism software is used to find out the absolute, relative IC50 value and hill slope value, ECOF finder software to find out the epidemiology cutoff value, WHONET 5.6 software analysis is used to find out resistant, susceptible or intermediate nature of commercially existing antibiotics. Size and structure of the newly designed antibiotics compare with the structure of commercially existing antibiotics and structure of organisms.

KEYWORDS: *MTT Assay, Cell Viability, IC 50, Agar Dis Diffusion, Graph pad ,EcoF Finder, EUCAST,NCCLS.*

1. INTRODUCTION

Throughout history, it has been a major worldwide problem to treat microbial diseases caused by bacteria and fungi due to impetuous development of resistance to antibacterial and antifungal drugs. In recent decades, the incidence of fungal infections has gone up all over the world. The development of new therapeutic agents is one of the essential goals in medicinal chemistry. Thiosemicarbazones have been investigated for medicinal studies for a long while due to their wide range of biological activities including antineoplastic, antimycobacterial, antibacterial, antifungal, antiviral, and antimalarial effects and versatility as nitrogen and sulfur donors allowing them to bring on a great variety of coordination modes. The organic crystals of thiosemicarbazone derivatives have high thermal stability and Non-linear optical properties. In addition thiosemicarbazone molecules containing π-electron conjugation system asymmetrized by the electron donor and acceptor groups are highly polarizable entities for NLO applications. Hence, In the present study the preparation, growth and Anti-Microbial efficiency of thiosemicarbazone derivatives of 2 Chloro benzaldehyde crystals is reported.

2. EXPERIMENTAL DETAILS

To a hot solution of 1.82 g of thiosemicarbazide dissolved in a 160 ml of methanol, a solution of 2.8114 g of 2-Chlorobenzaldehyde dissolved in 70 ml of methanol stirred for 30 minutes. The aggregate became stirred and refluxed for 4 hours. Then it become filtered and the filtrate become concerned with 1/2 the volume. The saturated solution was kept in rest and the beaker becomes blanketed with polythene paper. Some holes had been made on the polythene cover to facilitate sluggish evaporation. By means of adopting the

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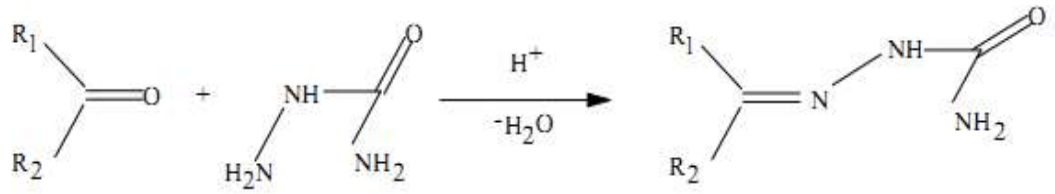
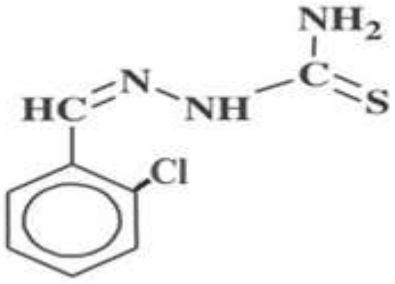
solution growth technique crystals of thiosemicarbazone of 2-Chlorobenzaldehyde have been grown from the supersaturated solution at room temperature. Crystals were gathered by way of filtration, washed with cold ethanol and dried in desiccators. These crystals had been suitable for characterization studies.Similarly other two compounds were prepared and recrystallization with suitable reagent and solvent respectively. It was characterized by FT-IR Study,UV-Study,NMR Study (^{1}H NMR AND ^{13}C-NMR),X-Ray Diffraction Studies ,TGA Study,NLO Study.





3. ANTIMICROBIAL STUDY

3.1. Disc Diffusion Test-Preparation of Mueller-Hinton Agar:

Mueller-Hinton agar need to be prepared from a commercially available dehydrated base, according to the manufacturer's instructions. Right away after autoclaving, allow it to chill in a 45 to 50^{o} C water tub. Pour the freshly prepared and cooled medium into glass or plastic, flat bottomed petri dishes on a degree, horizontal surface to give a uniform depth of approximately 4 mm. This corresponds to 60 to 70 ml of medium for plates with diameters of 150 mm. The agar medium has to be allowed to cool to room temperature and, until the plate is used, stored in a refrigerator (2 to 8^{0} C). Plates need to be employed within 7 days after cooling with the aid of incubating at 30 to 35^{0} C for 24 hours or longer.

3.2. Preparation of antibiotic stock solutions.

Stock solutions are prepared by formula
(1000/P) x V x C=W,
in which,P = efficiency of the antibiotic base, V=volume in ml required, C=final concentration of the solution and W=weight of the antimicrobial to be dissolved in V.

3.4. Disc diffusion strategies

The Kirby-Bauer and Stokes' strategies are typically used for antimicrobial susceptibility testing, with the Kirby-Bauer technique being endorsed by the NCCLS. At least 3 to 5 well-isolated colonies of the same morphological type are decided on an agar plate subculture. The pinnacle of each colony is touched with a loop, The boom is transferred into a tube containing 4 to 5 ml of a suitable broth medium, along with tryptic soy broth. The broth subculture is incubated at 35^{o}C till it achieves or exceeds the turbidity of the 0.5 McFarland wide spread (typically 2 to 6 hours)

3.5. Analyzing Plates and deciphering results

After 16 to 18 hours of incubation, each plate is tested. If the plate turned into suitably marked and the inoculum become accurate.The diameters of the zones of entire inhibition (as judged by way of the unaided eye) are measured, including the diameter of the disc. The sizes of the zones of inhibition are interpreted and the bacteria are recommended as Susceptible, intermediate, or resistant to the antibiotic agents which have been examined.

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3.6. MIC- Minimal Inhibitory Concentration

MIC

The MIC is the lowest concentration of antimicrobial agent that absolutely inhibits colony formation. If there's no growth at lower concentration however there is an increase at higher concentrations.

MIC Break Point

The concentration of the antibiotic at which maximum inhibition of bacteria is called MIC breaking point Susceptible (S) MIC < breakpoint of the given organism. Likely to be effective > 90% of the time.Intermediate (I) May be powerful at better doses or if antimicrobial concentrates. Resistant (R) MIC > breakpoint of the organism. Not going for you to achieve powerful degrees of the drug at secure doses.

3.7. IC50 and IC90 values

After the MIC values are study, further analysis of the inhibitory concentration at 50% (IC50) and 90% (IC90) of the bacterial lines are completed. Basically, the IC50 or IC90 is used to determine the dosage treatment of antimicrobial agent for in vivo medicine towards the pathogen inside the area to recognize the effectiveness of each drug. The method used to decide IC50 and IC90 values is Graph pad prism software

Formula:

$$IC_{50} = \frac{A+B}{2} : \text{ when } A = \frac{50 \times \text{MIC value of the next \% lower than 50\%}}{\text{the next \% lower than 50\%}}$$

$$B = \frac{50 \times \text{MIC value of the next \% higher than 50\%}}{\text{the next \% higher than 50\%}}$$

$$IC_{90} = \frac{C+D}{2} : \text{when } C = \frac{90 \times \text{MIC value of the next \% lower than 90\%}}{\text{the next \% lower than 90\%}}$$

$$D = \frac{90 \times \text{MIC value of the next \% higher than 90\%}}{\text{the next \% higher than 90\%}}$$

3.8. MTT Assays Study

A. Cell culture

Bacteria had been cultured in liquid medium (DMEM) supplemented 10% Fetal Bovine Serum (FBS), a 100 ug/ml antibiotics, and maintained under an environment of 5% CO_2 at 37ºC.

B.MTT assays

Assays which allow for the quantitative dimension of cellular loss of life in the course of cell subculture are important to any test involving variable strains or ex vivo cell clinical samples. Plate 1,000-100,000 cells in step with properly in a 96-nicely plate and incubate with an appropriate incentive for the desired time (generally 6-48 hours). Take away medium and wash cells with PBS. Upload MTT made up in medium to a very last concentration of 0.5 mg/mL.

Incubate for 30 minutes to 4 hours at 37°C, until intracellular crimson formazan crystals are seen below microscope. Put off MTT and upload solubilizing solution and tritrate. Incubate at room temperature or 37°C for 30 minutes to 2 hours, till the cells have listing and red crystals have dissolved. Measure absorbance at 570 nanometer

3.9. Application of Computers in Antibacterial Susceptibility Testing

A.WHONET 5.6 Software

Whonet 5.6 software was utilised to find out the http://www.Who.Int/emc/WHONET/instructions.Html This Programmed is beneficial in supplying cutting-edge recommendations, protocols to nearby laboratories, in figuring out the clusters of resistant isolates and rising outbreaks

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B.Graph Pad Prism 8 Software

Graph Pad Prism 8 Software was utilised to find out the IC 50 and EC 50 Value by the Nonlinear Regression Methods.

C.Test ECOF Finder

Test ECOF Finder was utilised to find out the ECV for the drug against bacteria's and to find out the drug wild types are not non wild types.

4. RESULTS AND DISCUSSION

4.1. Antimicrobial Activites

The existing antibiotics for this organism Streptococcus aureus are Methicillin, Cefoxitin, cefixime and oxacillin. Their MIC break point is obtained from EUCAST and NCCLS data base. Their Susceptible MIC break points are >2 mg/l , >4mg/l ,<0.5 mg/l and >2 mg/l respectively shown in the TSC2CB has optimum MIC break point 16 mg/l or 160 µg/ml.

4.2. MTT ASSAY TEST

S. No	Tested Sample Concentration (µg/ml)	Cell viability (%) (In Triplicates)			Mean Value (%)
1.	Control	100	100	100	100
2.	100	38.73	39.82	44.2	40.91
3.	90	49.45	50.32	46.38	48.71
4.	80	50.76	51.2	53.82	51.92
5.	70	58.86	55.57	58.42	57.61
6.	60	62.8	55.79	60.17	59.58
7.	50	65.42	61.7	62.8	63.3
8.	40	65.64	63.67	64.77	64.69
9.	30	77.24	67.83	66.52	70.53
10.	20	77.46	79.21	73.74	76.8
11.	10	94.09	94.09	94.09	91.6
12	5	98.68	99.12	87.74	95.18
13	2.5	99.56	99.78	89.93	96.42
14	1.25	96.93	97.81	89.49	94.74
15	0.625	91.90	96.49	88.18	92.19

Table: 1.1

IC50 values is the amount of drug leads to inhibit the half of targeted bacterias it is manually calculated by using the formula it was found to be 0.83 µg/ml.Graphpad prism 8 software is utilized to find the IC50 value and to draw the curve between cell viability % and log c [µg/ml]. The IC50 absolute value was 0.83 µg/ml and relative IC50 range is between 0.76 and 0.91 ug/ml. This is <1 µg/ml. This IC50 is optimal for good drugs. The hill slope value of the curve is –ve indicates the +ve inhibition of Streptococcus

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aureus by TSC2CB derivative. The IC 50 values of existing antibiotics Methicillin,Cefoxitin,Cefixime,Oxacillin against this bacteria are also lesser than 1 µg/ml.

4.3. GRAPPAD PRISM 8 ANALYSIS

log(inhibitor) vs. normalized response -- Variable slope	
Best-fit values	
Hill Slope	-0.9556
IC50	0.83
95% CI (Profile like lihood)	
LogIC50	1.883 to 1.960
Hill Slope	-1.083 to -0.8413
IC50	0.7643 to 0. 9128
Goodness of Fit	
Degrees of Freedom	37
R squared	0.9528
Sum of Squares	643.7
Sy.x	4.171

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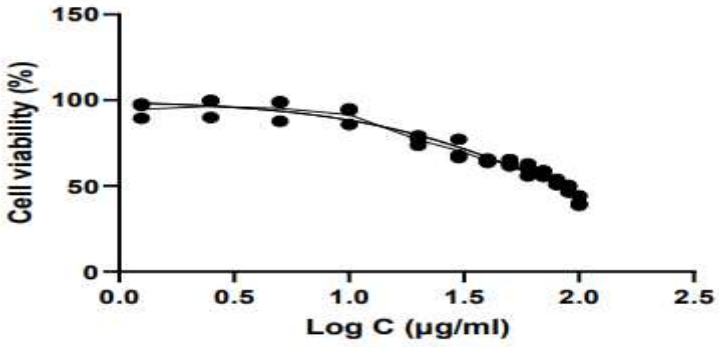
Sum of Squares	643.7
Sy.x	4.171
Replicates test for lack of fit	
SD replicates	3.898
SD lack of fit	4.754
Discrepancy (F)	1.487
P value	0.1957
Evidence of inadequate model?	No
Number of points	
# of X values	39
# Y values analyzed	39

Table:1.2

4.4. HILL SLOPE CURVE



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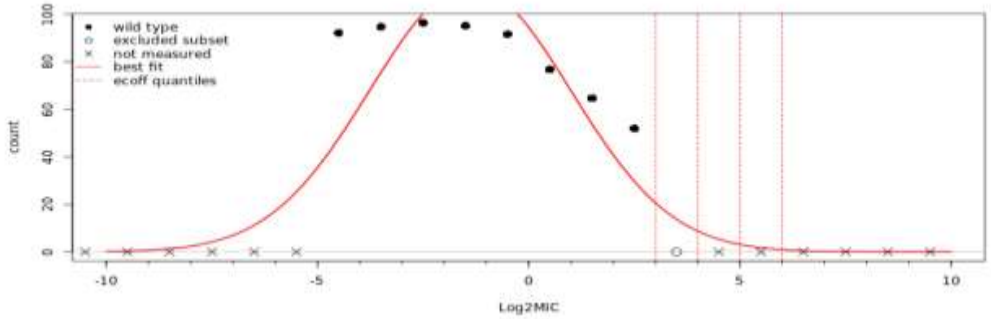
4.5. MIC AND MIC BREAK POINT

S. No	Tested sample concentration (µg/ml)	Cell Viability (%) Mean Value
1	160	0
2	150	0
3	140	0
4	130	0
5	120	7.5
6	110	22.8
7	100	40.91
8	90	48.71
9	80	51.92
10	70	57.61
11	60	59.58
12	50	63.3
13	40	64.69
14	30	70.53
15	20	76.8
16	10	91.6
17	5	95.18
18	2.5	96.42
19	1.25	94.74
20	0.625	92.19

Table:1.3

4.6. DENSITY CURVE



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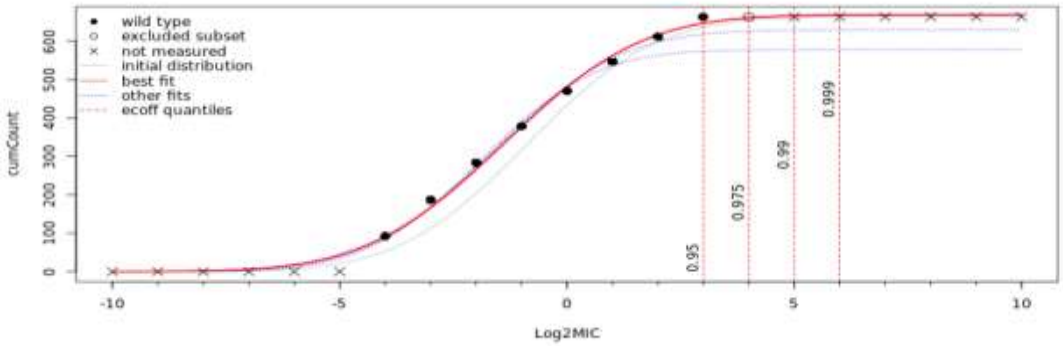
4.7. CUMULATIVE CURVE



4.8. EPIDEMIOLOGICAL STATISTICAL DATA

```
mean          sd          K
 -0.8798064   2.2242261 663.5400000
Search concentration:  1 2 3 4
```

Formula: cumCount ~ fnorm(conc, mean, sd, K)

Parameters:

	Estimate	Std. Error	t value	Pr(>|t|)	
mean	**-1.3846**	**0.1582**	**-8.754**	**2.75e-06**	***
sd	**2.3861**	**0.1769**	**13.487**	**3.47e-08**	***
K	**668.8193**	**22.4319**	**29.816**	**7.13e-12**	***

Signif. codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

Residual standard error: 18.12 on 11 degrees of freedom

Number of iterations to convergence: 8

Achieved convergence tolerance: 2.019e-06

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```
Parameters:
     Estimate Std. Error t value Pr(>|t|)
mean -1.3846    0.1582  -8.754 2.75e-06 ***
sd    2.3861    0.1769  13.487 3.47e-08 ***
K   668.8193   22.4319  29.816 7.13e-12 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 18.12 on 11 degrees of freedom

Number of iterations to convergence: 8
Achieved convergence tolerance: 2.019e-06

---
ECOFF quantiles:
 Q_0.95 Q_0.975  Q_0.99 Q_0.999
   0.08    0.16    0.32    0.64
```

4.9. WHONET 5.6 SOFTWARE ANALYSIS

By the application of whonet 5.6.software, we had analysed the action of existing antibiotics against the Streptococcus aureus bacteria. Type of the sample is blood and number of isolates utilized is one. The following antibiotics, Which were analysised against the organism Streptococcus aureus and their class, sub class, code, 110 method, break point, range of susceptibility, isolate number, % resistant, % intermediate are this case all antibiotics are resistant because their MIC break point lower than the MIC values, They all are less effective against this organism but the TSC2CB has lower MIC than MIC break point therefore it is susceptible.

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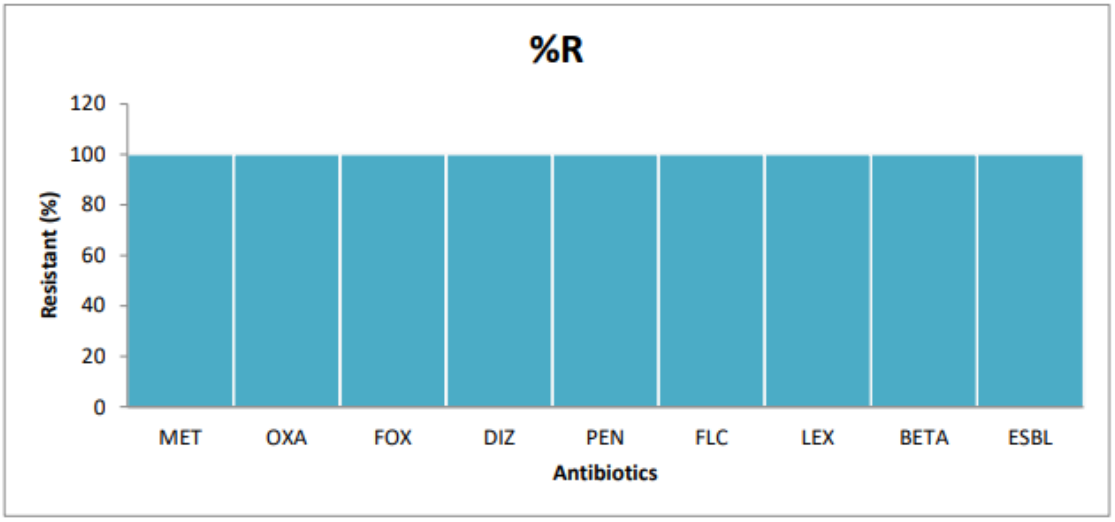
4.9.(a). WHONET OUT PUT DATA

Code	Antibiotic name	Antibiotic class	Antibiotic subclass	Code	Methods	Break Points	Num Ber	%R	%I	%S	%R 95%C.I.	Num ber
MET_ND5	Methicillin	Penicillins	Penicillin (Stable)	MET	Disk	10 – 13	1	100	0	0	5.5-100	0
OXA_ND10	Oxacillin	Penicillins	Penicillin (Stable)	OXA	Disk		1	100	0	0	5.5-100	0
FOX_ND10	Cefoxitin	Cephems	Cephamycin	FOX	Disk		1	100	0	0	5.5-100	0
DIZ_ND10	Cefodizime	Cephems	Cephalosporin III	DIZ	Disk		1	100	0	0	5.5-100	0
PEN_ND10	Penicillin G	Penicillins	Penicillin	PEN	Disk	S >= 29	1	100	0	0	5.5-100	0
FLC_ND	Flucloxacillin	Penicillins	Penicillin (Stable)	FLC	Disk		1	100	0	0	5.5-100	0
LEX_ND30	Cephalexin	Cephems-Oral	Cephalosporin	LEX	Disk		1	100	0	0	5.5-100	0
BETA_LACT	Beta-lactamase			BETA			1	100		0		
ESBL	ESBL			ESBL			1	100		0		

Table:1.4

4.9.(b). WHONET RESISTANT % OUTPUT



5. AGAR DISK DIFFUSION STUDY

Petri dish plates are used to find out the inhibition zone width of the antibiotics TSC2CB against Streptococcus aureus. The optimum antibiotic concentration for this TSC2CB is 2.5µg/ml. The maximum inhibition zone Streptococcus aureus organism at 2.5µg/ml concentration of TSC2CB antibiotic is shown in the level 16 respectively. As per the EUCAST and NCCLS database the zone with >26 mm are Susceptible.There fore our new antibiotic TSC2CB is Susceptible against the bacteria Streptococcus aureus. From the above discussions the derivative TSC2CB was proved as a better antibiotic against Streptococcus aureus bacteria than the existing antibiotics by in vitro study and it is susceptible against the diseases, skin infection, pimples, high fever, boils cellulitis,,Kindney Damage, Toxic shock syndrome, bacteremia, meningitis,

Agar Disk Diffusion Methods (Inhibition Zone With)
Staphlococcus Aureus-Concentration of Antibiotic 2.5µg/ml

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Volume: 8 | Issue: 6 | June 2023 - Peer Reviewed Journal

TSC2CB (16 mm)

6. SUMMARY AND CONCLUSION

In vitro method of analysis is base for new drug designing from novel derivatives of organic and semiorganic NLO compounds are recently being entered into this field. Thiosemiccarbozones are having enormous medicine applications due to the presence of hetero atoms nitrogen “N” and sulphur “S” in its molecular structure. The carbonyl compounds are also having the large clinical applications to cure many diseases. We merge these two classes of organic compounds and their substitution by solution growth techniques of crystal growth and tested as antibiotics against few fastidious and non fastidious gram +ve and gram –ve organisms. From the above antimicrobial following conclusions were made, the minimum inhibition concentration (MIC) values is very low for the organism Escherichia coli (0.625 µg/ml) against TSC2CB. The MIC break point range is between 110µg/ml and 130µg/ml in all the cases MIC break points are not equal to MIC but greater than MIC values. Therefore we concluded that susceptible type of inhibition shown by all the new compounds. The half inhibitory maximum value (IC50) was also calculated by the graph pad prism software, all the TSC derivatives have the IC50 value lesser than one (<1) (0.7 to 0.9 µg/ml) indicated that all the prepared one TSC derivatives are efficient against the above said one organisms. The hill slope value of curve between the cell viability vs log (c) is negative for all the one TSC derivatives obtained from graphpad prism software concluded that the inhibition capacity of the one TSC derivatives are susceptible against the tested organisms. The WHONET Study results reveal that all the Existing antibiotics in medicals against the above mentioned bacteria (Organisms) are having somewhat poor than the TSC derivatives. Structures and Size of all Existing antibiotics in medicals against the above mentioned bacteria(Organisms) are having comparatively larger shape and size than the TSC derivatives, Therefore, They Lost their Surface area and enter in to and Exit from bacteria is difficult for existing antibiotics when compare with TSC derivatives. The epidemiology cut off value (ECV) for all the one TSC derivatives against all the organisms are found around 0.08 (95%) µg/ml in all the one case the MIC values are higher than there ECV value therefore organisms tested in this study are not belonging to wild type distribution against all the one TSC derivatives. Hence there are no chances for failure in the clinical treatment is confirmed.

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