Antimicrobial Evaluation of Dichloro Chromene Isoxazoles and Isoxazoline Derivatives

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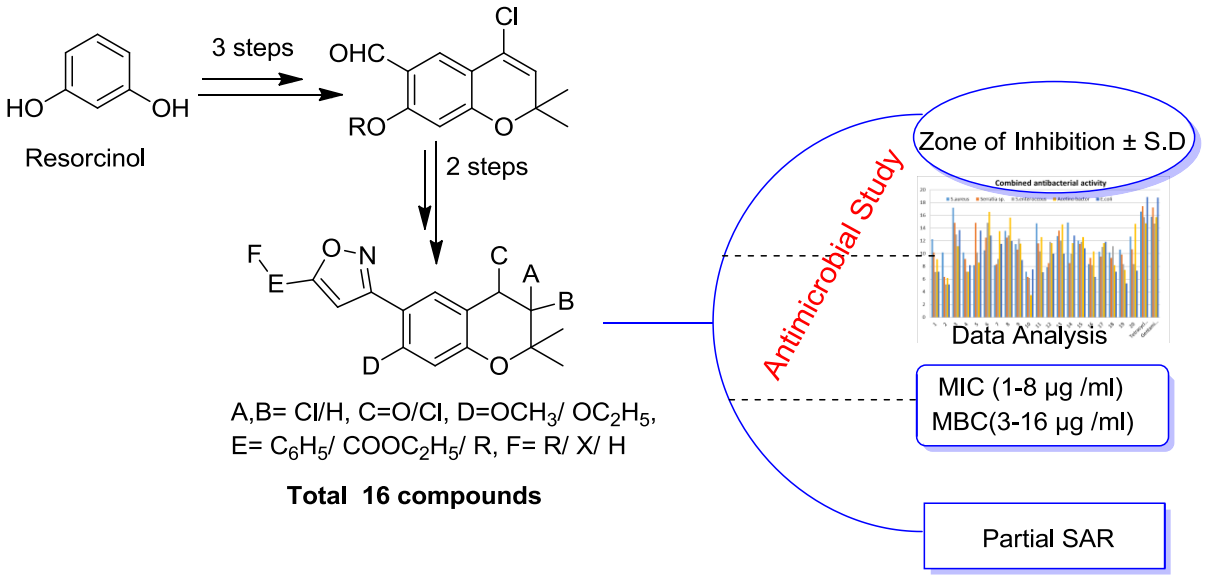
Abstract

In the present study, the synthesized benzopyran isoxazolines and isoxazoles were evaluated for antibacterial activity by Agar well diffusion method against multi drug resistance (MDR) clinical isolates of five bacterial strains.*i.e., Staphylococcus sp.* (2413), *Enterococcus sp*. (2449), and *Escherichia sp.* (2461), *Acinetobacter sp.* (2457) and *Serratia sp* (2442). MIC (Minimum inhibitory concentration) and MBC (Minimum bactericidal concentration) of the potent derivatives were calculated and tabulated. A partial structure activity relationship (SAR) was done on the basis of microbial inhibition data. Compounds **3**, **6**, **8**, **9**, **11, 14** and **20** were the most promising antibacterial molecule. Compound **3,** the precursor of isoxazole / isoxazoline found to be most active antibacterial candidate as well as bactericidal too.



Keywords: Benzopyran Isoxazoles, Antibacterial Assay, MIC, MBC, SAR

1.0 Introduction

Over the preceding years in our understanding of human disease we have made a rapid progress in the field of antibiotics. Getting a novel antibiotic seems to be hard all these days. The cause behind it may be more often bacteria are exposed to a certain antibiotic, the more opportunities they have to evolve resistance to combat it. This furnishes antibiotics, the authentication of modern medicine, nearly ineffective against 'nightmare bacteria'. The resistance rates in the '90s were at 10 to 15 percent while, now it's up to 60 percent in hospitals.[1] Therefore, there is a pressing need of communal feat to address the menace and simultaneously preface of new drug candidates to scrap these pandemic infections. An additional major concern for antibiotic

resistance is that it has not been represented appropriately across the world. Statistical data shows that in low- and middle-income countries, where antibiotic resistance is a serious problem but rarely the focus of policy solutions.[2] The resistance of E.coli is rising day by day in many world regions, according to resistance map.[3] India has the highest rates of resistance to all the antibiotics as compared to all other countries throughout world; E. coli strains are more than 80 percent resistant to different classes of drugs, which clearly indicates treatment options are becoming increasingly limited day by day.[4] Hence, in view of the latest trend of bacterial resistant the screening of the synthesised compounds were conducted against ampicillin and gentamicin resistant *Escherichia coli* and *Staphylococcus aureus*; ciprofloxacin resistant *Serratia*s pecies;imepenem resistant *Acinetobacter* and *Enterococcus* (MDR) species.

Flavones/chromenes/Benzopyran are reputed for antibacterial activity from ancient days both synthetic as well as natural origin. Literature says flavones having benzimidazole scaffolds has good antibacterial property against *Staphylococcus aureus* (Fig. 1).[5]

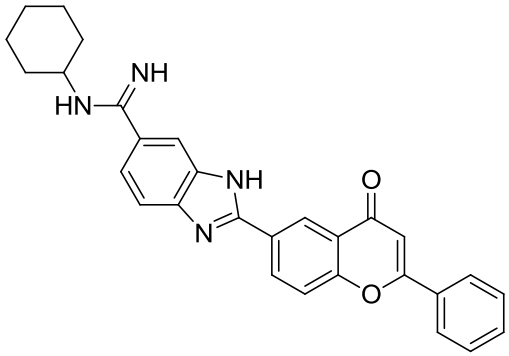


Fig.1. Chromene with imidazole having antibacterial activity against *Staphylococcus aureus*

Natural flavones such as semiglabrin, pseudosemiglabrin, apollinine, lanceolatin A, crysin isolated from *Tephrosia nubica* were very effective against different strains of bacteria viz *Penicillium funiculus, Fusarium moniliforumspergillus, Aspergillus niger* etc (Fig.2).[6]

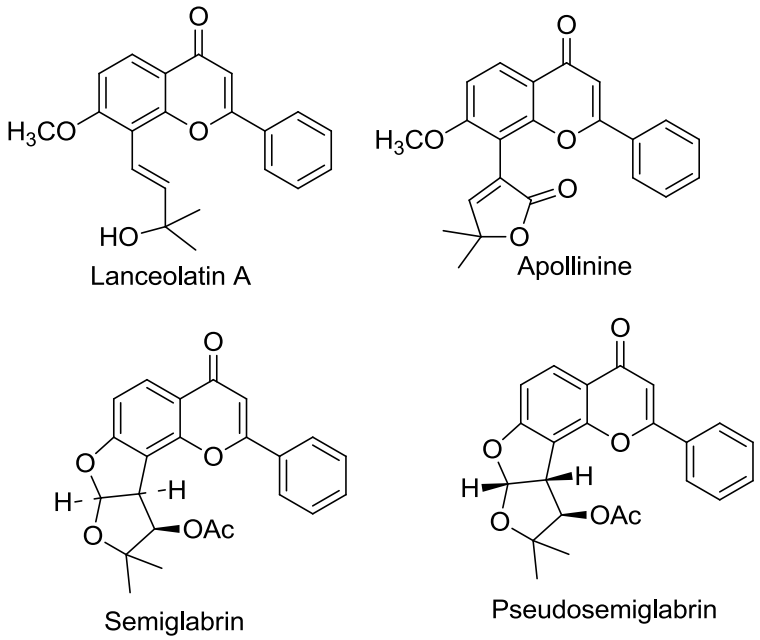


Fig.2. Some examples of natural chromene as antibacterial agents

Isoxazoles and isoxazolines are ubiquitously found in nature as well as synthesized from various precursors.[7] It has been used as FDA approved drugs in pharmaceuticals, such as antibiotics (cloxacillin, dicloxacillin, cycloserine, etc).[8] chromenes fused with these scaffold are also known as antimicrobial agents when we look at the literature precence.[9] But Chromene isoxazoles/ isoxazolines having halogen substituents (monochloro, geminal chlorine or vicinal chlorine) was never reported earlier as any antibacterial agents. It could be used to enhance the activity as efficiency of substrate target binding ameliorates.[10] Shah and co-workers has reported the presence of chlorine accentuates the antibacterial properties of isoxazole and isoxazoline.[11] In this report we have focused on antimicrobial evaluation of our synthesized dichloro chromene isoxazoles. With the skilfulness in the field of medicinal chemistry, [12] we have synthesized different chromene isoxazoles and the isoxazolines in our previous paper.[13] This is an extension of that paper where we have attempted a biological study (antimicrobial) of those synthesized compounds and analysed there efficacy with reference to standard drugs like Tetracycline and Gentamicin.

2.0 Materials and methods

2.01. Chemistry

^{1}H and ^{13}C NMR spectra were recorded on 400 and 500 MHz spectrometers with TMS as the internal standard. Chemical shifts are expressed in parts per million (δ ppm). Silica gel coated aluminium plates were used for TLC. The products were purified by column chromatography on silica gel (60-120/100-200/230-400 mesh) using petroleum ether–ethyl acetate as the eluent to obtain the pure products. Reagents used were mostly purchased from Sigma Aldrich if not mentioned otherwise.

2.02. Biology

2.02.1. Microorganisms and culture media

The following five MDR bacterial clinical isolates were used in this study: *Staphylococcus aureus* (2413), *Enterococcus* sp. (2449), *Serratia sp*. (2442), *Acinetobacter* sp. (2457) and *Escherichia coli* (2461). All the strains were obtained from Dr. Kumardeep Dutta Choudhary, Department of Medical Oncology, Rajiv Gandhi Cancer Research Institute, Delhi, India with their respective antibiotic resistance profiles (provided in below table). All bacterial strains were grown on 5% nutrient broth (NB). Following the initial incubation, organisms were suspended in 15ml of NB and optical density readings were compared to a 0.5 McFarland standard. For the MIC determination, bacterial suspension of 5×10^{6} colony-forming units (cfu) ml was employed [CLSI, Performance Standards for Antimicrobial Disk Susceptibility Tests, Approved Standard, 7th ed., CLSI document M02-A11.]

2.02.2. Agar well diffusion assay

Antibacterial activity of the synthesised compounds was determined by agar well diffusion method by previously described method by Rosa et al. Briefly, nutrient agar plates were inoculated with 0.1 mL of each organism ($1x10^{8}$ CFU/mL). Subsequently, wells of 6 mm size were bored into the nutrient agar set plates containing the bacterial culture and treated with 40 µL of samples prepared from a stock solution of 1mg/mL. in 2% DMSO solution. The plates were incubated at 37°C for 24 h. Standard antibiotic disc of tetracycline, gentamycin (30 µg) and 2% DMSO solution was taken as positive and negative as control. Assays were carried out by triplicate and the antimicrobial activity was expressed as the mean diameter of inhibition zones (mm) with standard deviation.

2.02.3. Determination of Minimum Inhibitory concentration (MIC) and Minimum Bactericidal concentration (MBC)

MIC was determined by microdilution method described by Weigand *et al*. Briefly, the cultures were diluted in Mueller-Hinton broth at a density of 0.5 McFarland turbidity. 0.5 mL of a bacterial suspension (5×10^{6} CFU/mL)

was added to 4.5 mL of susceptibility test broth containing diluted compound solution, prepared by serial two-fold dilution from the stock solution (1mg/mL). A number of wells were reserved in each plate for control of sterility (no inoculum was added), inoculum viability (no sample solution was added) and DMSO inhibitory effect. The plates were then incubated for 24 h at 37° C. After 24 h of incubation, the absorbance was read at 570 nm in ELISA reader. MIC of tetracycline was used as standard determined in parallel experiment for comparison. Assays were carried out by triplicate and MIC was considered as the lowest concentration of the sample that prevented visible growth.

2.02.4. Determination of Minimum Bactericidal concentration (MBC)

The MBC assays were performed as described by Goda *et al*. Briefly, the wells which showed complete absence of bacterial growth were identified and aliquots (10µl) of each well were transferred to Muller Hilton agar plates and incubated at 37ºC for 24 hrs. Experiments were carried out by triplicate and the complete absence of growth was considered as the minimum bactericidal concentration.

2.02.5. Statistical Analysis

All experiments were carried out in triplicates and statistical analysis was carried out using Graph Pad Prism version 4.00 for Windows, GraphPad Software, San Diego California USA. Data are presented as Mean±SD.

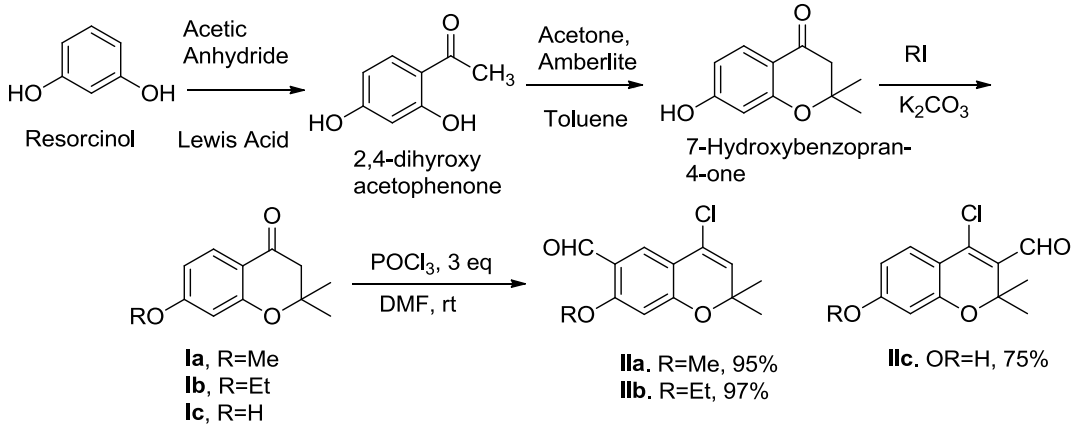
2.02.6. Graphical Analysis

All experiments were analysed using "Microsoft Office Excel 2007", column charts to analyse the antimicrobial evaluation.

2.03. Result and Discussion:

2.03.1. Chemistry

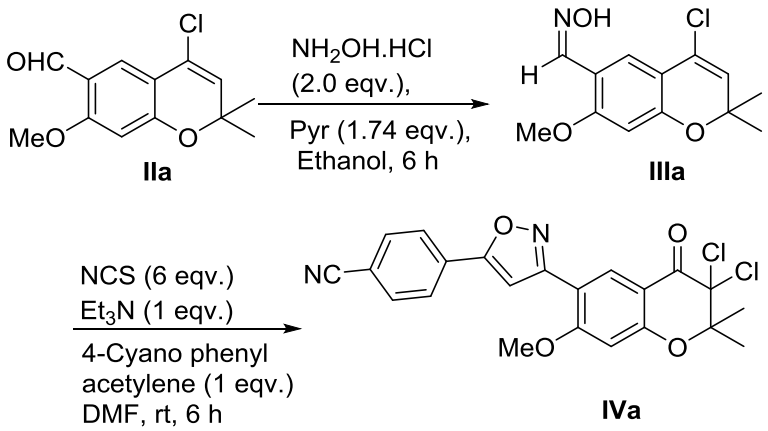
For the synthesis, our strategy began from 4-chloro-6-formyl benzopyran **II** synthesis. This was derived from chromanone **I** which was easily achieved from resorcinol by acylating it (Friedel Craft's reaction) and then condensation with substituted ketones (Aldol condensation). Then the 7-hydroxy benzopyran-4-one so formed on alkylation yield 7-alkoxy-2,2-dimethylchroman-4-one **I**. By treating **I** with Vilsmeier-Haak reagent, we could affect formylation at C-6 as well as chlorination at C-4 of the benzopyran (Scheme-1).



Scheme 1: Synthesis of 4-chloro-6-formyl benzopyran

Formylated benzopyran **IIa** was converted to corresponding oxime **IIIa** by treating with hydroxyl amine hydrochloride and pyridine (scheme 3). Benzopyran aldoxime derivative **IIIa** thus obtained was treated in DMF

with 4-CN-phenyl acetylene as dienophile and NCS in the presence of triethylamine at room temperature to obtain desired benzopyran isoxazole compound **IVa** in 6 hrs at room temperature (scheme 2).



Scheme 2: Synthesis of chromene isoxazole hybrid from chromene carbaldehyde

While characterizing **IVa**, 1H spectroscopy the proton shifts for dimethyl appeared at C2 (· 1.72), methoxy signal appeared at · 4.00, isoxazole proton signal appeared at · 7.06. All the aromatic proton was clear (see supporting). Dimethyl signal appeared like a hump instead of sharp peak (Fig. 3). When 13C spectra was analysed we achieved a quaternary carbon (carbonyl carbon C4) at · 178.61 (fig.4) instead of chlorine (· 125.99) and a loss of olefin shift at · · 125.35 in chlorochromene (see supporting).

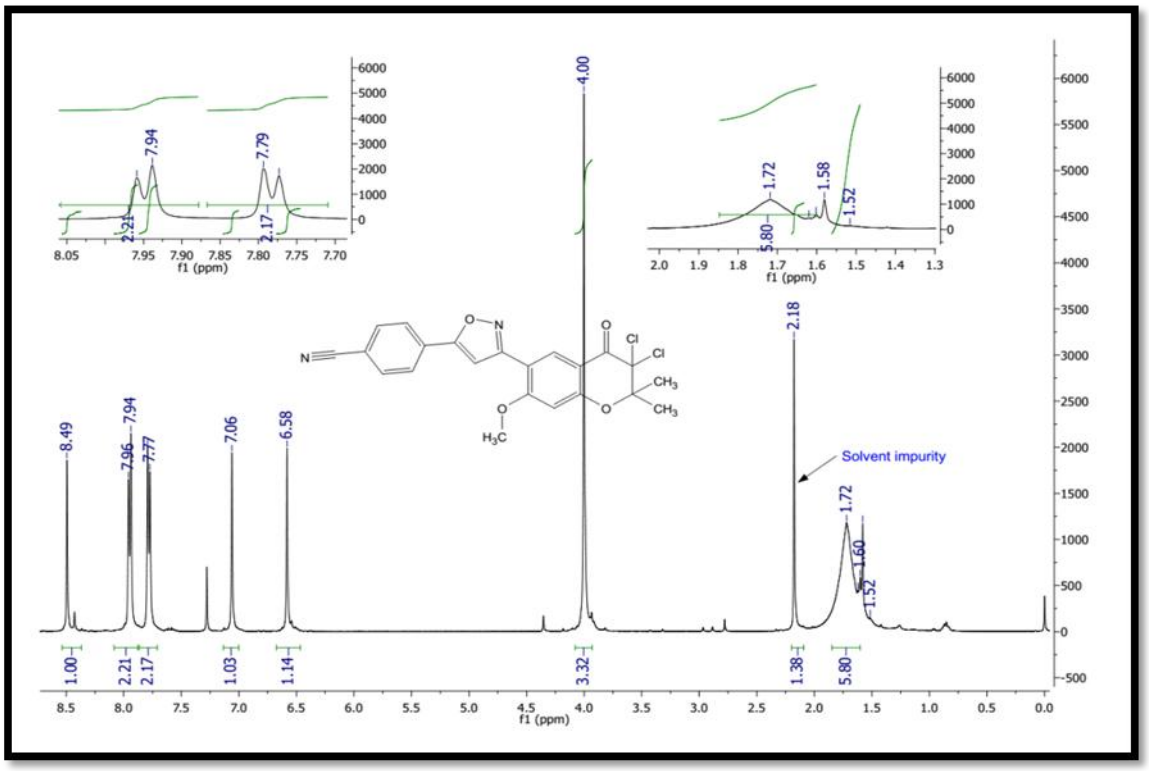


Fig.3: 1H NMR spectra of **Iva**

After the characterization of the final compound we have synthesized 16 derivatives. Structures are given in Table1 (**05**-**20**). A detailed discussion of all the synthesized compounds was done in the previous paper.[13]

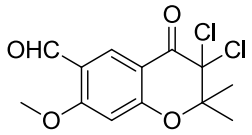
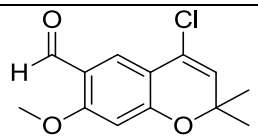
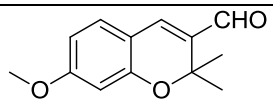
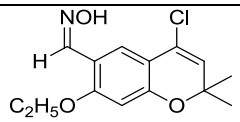
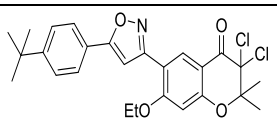
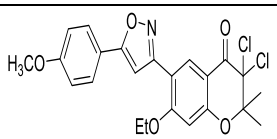
2.03.2. Biology

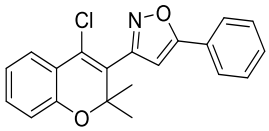
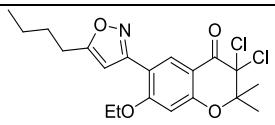
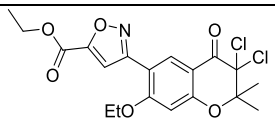
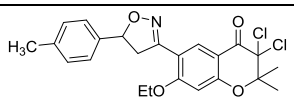
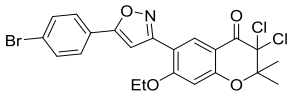
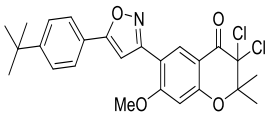
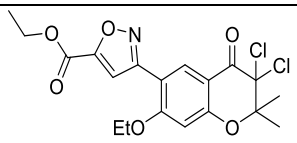
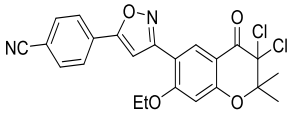
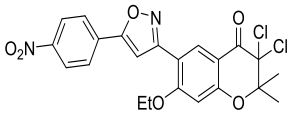
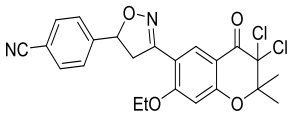
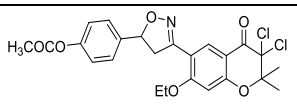
2.03.2.1. Antibacterial activity

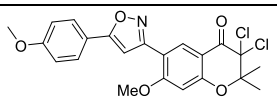
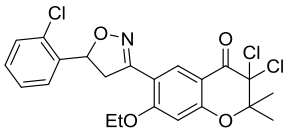
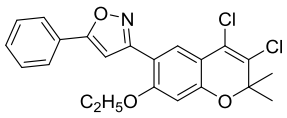
In order to test the antibacterial property of the synthesised compounds, agar well diffusion assay was performed. The antimicrobial drugs, tetracycline and gentamycine, were used as positive controls. The antibacterial activity of all the compounds were evaluated in-vitro by measuring the inhibition zone (in mm) for growth inhibitory effect against Multi drug resistance (MDR) clinical isolates of five bacterial strains; two Gram-positive bacteria (*Staphylococcus aureus* (2413), *Enterococcus sp.* (2449),) and three Gram-negative bacteria (*Escherichia coli* (2461), *Acinetobacter sp.* (2457) and *Serratia sp* (2442).) The antibacterial activities were tested at concentrations of 1mg/ml in 2% DMSO solution. The bacterial cultures were inoculated and incubated according to the described protocol of the experimental section.

All the synthesized molecules and their precursors were analysed for an antibacterial assay. The experimental results of this *in vitro* antibacterial evaluation are summarized in Tables 1.

(Table 1) In vitro antibacterial activity of the benzopyran fused isoxazole and isoxazoline derivatives against different bacterial strains

S. No	Name of the compound	*S. aureus*	*Serratia sp*	*S. enteroccous*	*Acinetobactor*	*E.coli*
01		12.236±0.20	10.165±0.28	7.126±0.13	9.105±0.28	7.160±0.38
02		10.166±0.20	6.330±0.13	5.166±0.28	6.166±0.26	5.166±0.28
03		17.166±0.28	14.83±0.62	13±0.50	11.146±0.22	13.66±0.47
04		10.166±0.28	9.16±0.23	7.166±0.28	7.160±0.36	8.176±0.28
05		8.16±0.23	14.83±0.62	10.16±0.23	8.56±0.18	13.66±0.47
06		10.48±0.54	12.50±0.40	14.83±0.62	16.548±0.23	12.83±0.23

S. No	Name of the compound	*S. aureus*	*Serratia sp*	*S. enteroccous*	*Acinetobactor*	*E.coli*
07		8.16±0.23	8.33±0.47	9.16±0.23	13.501±0.23	11.50±0.40
08		13.547±0.20	12.501±0.40	12.832±0.23	15.657±0.51	12.010±0.00
09		11.475±0.10	10.60±0.40	12.357±0.30	11.573±0.21	09.001±0.06
10		7.16±0.13	6.33±0.46	6.16±0.23	03.501±0.29	07.50±0.40
11		14.735±0.20	11.60±0.40	10.354±0.36	12.573±0.22	07.058±0.06
12		07.847±0.23	08.501±0.46	11.830±0.13	11.657±0.51	10.010±0.00
13		12. 745±0. 01	13.60±0.15	12.057±0.20	14.573±0.16	10.001±0.25
14		14.847±0.15	08.500±0.43	10.010±0.03	11.657±0.51	12.830±0.01
15		12.05±0.30	11.50±0.47	11.83±0.62	12.58±0.23	10.83±0.02
16		8.33±0.47	9.33±0.40	8.16±0.23	10.33±0.38	6.33±0.47
17		10.287±0.05	09.500±0.40	11.010±0.02	11.657±0.48	11.830±0.08

S. No	Name of the compound	*S. aureus*	*Serratia sp*	*S. enteroccous*	*Acinetobactor*	*E.coli*
18		10.166±0.26	09.330±0.13	11.166±0.28	8.166±0.20	07.166±0.18
19		10.62±0.43	09.84±0.23	8.33±0.47	07.44±0.15	5.33±0.31
20		12.66±0.63	10.66±0.23	8.33±0.47	14.66±0.15	7.33±0.28
	Tetracycline	16.56±0.58	17.42±0.58	15.71±0.58	14.73±0.35	18.85±0.53
	Gentamicin	15.76±0.58	17.23±0.58	14.68±0.35	15.71±0.58	18.79±0.58

N.B. Antimicrobial activity expressed as diameter of zone of inhibition in mm including 6 mm as diameter of the well. Value (mm) ±S.D of three replicates. All compounds were tested at 1mg/mL concentration. 30 µg Tetracycline and Gentamycin discs were used as positive control. 2% DMSO solution was used as negative control,

2.03.2.2. Data Analysis

We have made a comparison study of our synthesised compounds and their precursors with the help of "Microsoft Office excel 2007" for a transparent analysis against five different strains of bacteria.

The obtained result revealed that among the tested compounds **1**-**20** only **3**, **8, 11, 13** and **14** showed good inhibition against ***Staphylococcus aureus***. As a general study the formylated chromene **3** which is a precursor with no substitution at any position shows very good inhibitory effect (17.166±0.28), even more than the standard drug we have taken (16.56±0.58 &15.76±0.5). **11**and **14** (Ethoxy substituted at 7th position of benzopyran having isoxazoline and isoxazole ring with Br, CN with heterocyclic ring respectively). **8** and **13** shows less inhibitory effect but can be considered positively. These compound posses isoxazole ring with same ethoxide at 7th position of benzopyran. So, it can be concluded that the benzopyran compounds having 7ethoxy with isoxazole at aromatic part could be used positively against *S. aureus* (Fig. 4). When we screened our molecules **1**-**20** against ***Serratia sp***. strain, we found that compound **3 5** and **13** showed most promising inhibitory effect . Compound **3** which is a formylated benzopyran, compound **5;**a tertiary butyl group substituted isoxazole of 7 methoxy *gem* dichloro benzopyran and compound **13**;an ester derivative of *gem* dichloro 7-ethoxy benzopyran with an isoxazole moiety showing inhibition zones ranging between 13-15 mm indicating the presence of these groups or the pharmacophore is responsible for bacterial activity against this bacterial strain (Fig.4). According to our analysis report, it is seen that among all the testes compound **1-20, only 3, 5**, **6**, **8**, **9**, **13**, **15** showed good inhibition against *S.enteroccous strains* with inhibition zones ranging between 9-14 mm. Compound **6** which has a ethoxy group at 7th position of benzopyran and methoxy attached to isoxazole with a benzene linker has maximum antibiotic effect. Next, to it **5**, **8**, **9**, **13**, **15**, which all have ethoxy at 7th of benzopyran along with isoxazole are highly potent against this species. Compound **3** which is a formylated benzopyran also show good inhibitory effect (Fig.4).The experimental results show that among all the tested compounds **1**-**20**, Compound **6**, **8** and **20** shows very good inhibitory effect against *Acinetobacter* with inhibition zones ranging between 14-16 mm. Compound **6** and **8** which consist of isoxazoles with 7-ethoxide of benzopyran shows significant inhibition against this species, but the interesting

observation is that **8** does not possess an aromatic linker which means aromatic linker is not at all responsible for bacterial activity. Another vital observation was that **20** which is a vicinal dichloro compound of benzopyran is equally potent as tetracycline (Fig.4). Compounds **1**-**20** presented no significant inhibition zones against *E. coli* which gives us a strong affirmation that these classes of benzopyran isoxazoles compounds are totally beneficial for symbiotic bacteria. It is vague to give a single antibiotic against different types of bacterial infection. Still then if we look at the excel sheet graph of total antibacterial activity of the synthesized compound we can conclude that **3** and **6** can be good choice of treatment against various bacterial infections (Fig.4).

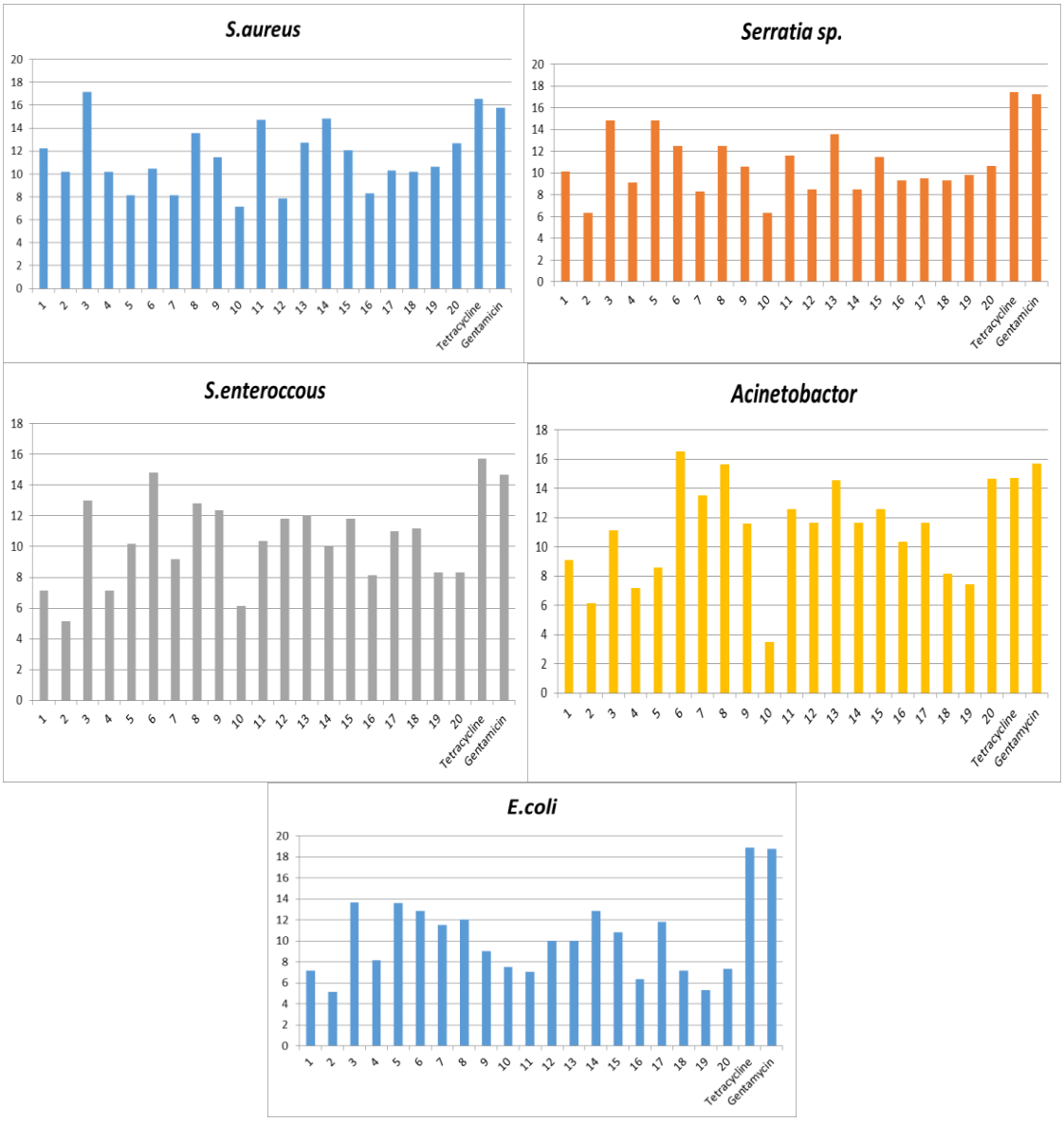


Fig.4. A combined graphical representation of antibacterial activity of 20 compounds against all the 5 strains discussed above

2.03.2.3. Minimum inhibitory Concentration (MIC) and Minimum bactericidal concentration (MBC)

From the above analysis we draw a conclusion that the compounds like **3**, **5**, **6**, **7**, **8**, **9**, **11**, **13**, **14**, **15**, and **20** shows appreciable antimicrobial effect. Hence we have chosen these compounds and proceeded for MIC and MBC studies. The results of the experiment is tabulated as follows (Table 2).

Table 2 MICs and MBCs expressed in µg/ml of the most active compounds against MDR resistance clinical isolates of bacterial strains

Compound	***S.aureus***		***Serratia***		***S. enteroccous***		***Acinetobactor***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
3	1.95	3.90	1.95	3.90	3.90	7.85		
5			3.90	7.85	7.81	15.62		
6					3.90	7.85		
7							1.95	3.90
8	3.90	7.85			1.95	3.90	1.95	3.90
9					3.90	7.85		
11	1.95	3.90						
13	3.90	7.85	7.81	15.62	3.90	7.85		
14	1.95	3.90						
15					3.90	7.85		
20							1.95	3.90
Tetracycline	0.5	0.8	1.2	2	1.87	3.75	1	2
Gentamycin	0.46	0.93	1.87	3.75	0.93	1.87	3.75	7.50

From the above study we conclude that for the most active compounds (**3**, **5**, **6**, **8**, **9**, **11, 13**, **14**, **15** and **20**) of he series, the MIC and MBC were evaluated against their corresponding bacterial strains and the results are presented in table 3. All of these compounds showed good antibacterial effect with the MIC values ranges in between 1-8 µg /ml. All the compound assayed showed bactericidal activity with the MBC value in the ranges of 3-16 µg /ml. Compound **3** found to be most effective antibacterial candidate as well as bactericidal too.

2.03.2.4. Structure activity relationship

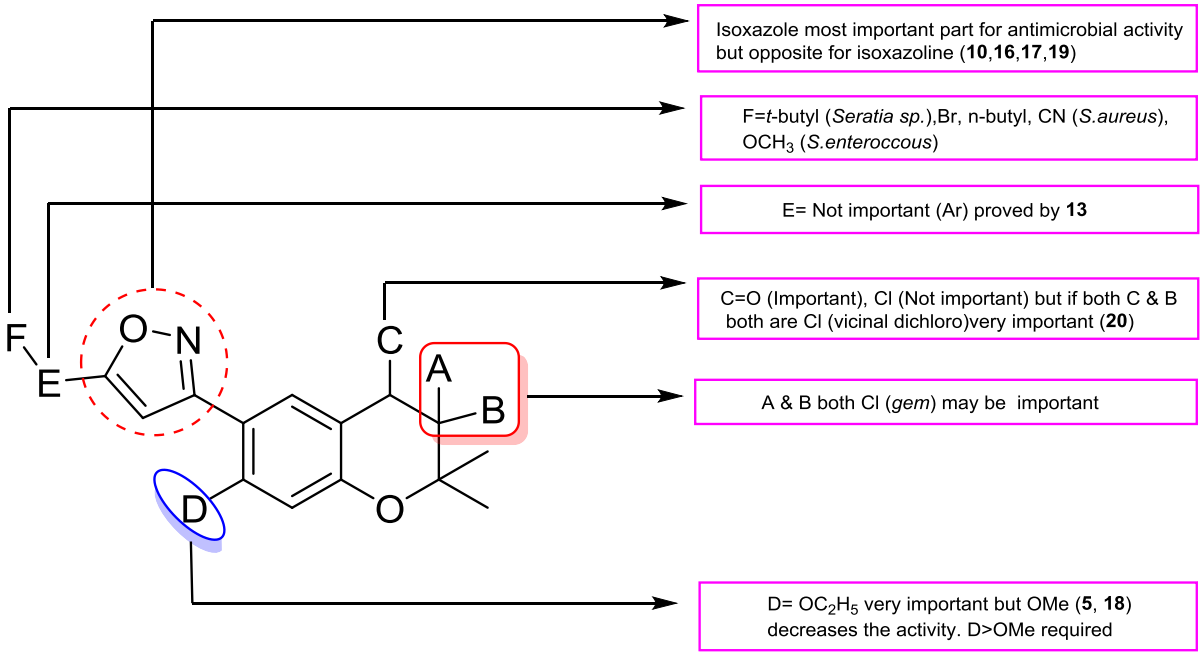


Fig.5 Structure activity relationship of 16 compounds with respect to inhibition of 5 bacterial strains

From the graphical analysis made from zone inhibition, MIC and MBC done above, some conclusions can be made regarding SAR are as follows

Isoxazoles are very important for antibacterial activity when attached to aromatic part of benzopyran or flavones moiety but isoxazolines are less important as we have observed less inhibition in case of isoxazoline containing molecules **10**, **16**, **17**, and **19** (Fig. 5).

If F (Fig.5) is an alkyl group such as *t*-butyl or n-butyl activity increases (**05**) but if F= alkyl but D= OCH3 then there is a decrease in activity. So D also should be greater than methoxy group. (Fig.5). If F is a CN or Br like withdrawing group we have observed an increase in activity (**11**, **14**, and **15**).

According to our experiment there is no requirement of a linker like benzene (E in Fig.5) as **13** shows an enhanced activity than others against four different strains of bacteria (Table 1).

A & B if are Cl (Geminal dichloro) may increase the activity because some molecules possessing *gem* dichloro lack this activity but if vicinal dichloro is present (**20** Table 1) C=Cl & B=Cl then a sharp increase in activity took place. But if a single Cl is attached C=Cl (**2**, **4** Table 1) there is a decrease in activity.

Precursor of the isoxazole which is a formylated benzopyran shows an excellent activity even more than standard drugs (**3** Table 1). Other starting compounds (**1**, **2**, and **4)** are not so effective. Hence our focus for the next study will be on **3** like starting molecules.

2.04. Conclusion

Our synthesized isoxazoles having dichloride (both geminal and vicinal) benzopyran shows a significant inhibitory effect against different strains of bacteria proved by in vitro antibacterial assays such as zone of inhibition, MIC (1-8 µg /ml), MBC (3-16 µg /ml). A comparison study of all the synthesized compounds was done with the help of Microsoft excels against 5 different strains. The graphical representation gave us an idea of a structure activity relationship. We have made detail SAR of all the molecules with relation to different

strains of bacteria. The vicinal dichloro isoxazole of benzopyran derivative is very much effective against S.aureus. Hence these chromene isoxazole scaffolds can act as good candidates against different class of bacterial infections after a clinical trial.

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