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SYNTHESIS, SPECTROSCOPIC STUDIES, ANTIBACTERIAL ACTIVITY OF CHALCONES AND COLORIMETRIC EVALUATION OF THE TIME-KILLING ASSAY FOR NEWLY SYNTHESIZED CHALCONES USING RESAZURIN**

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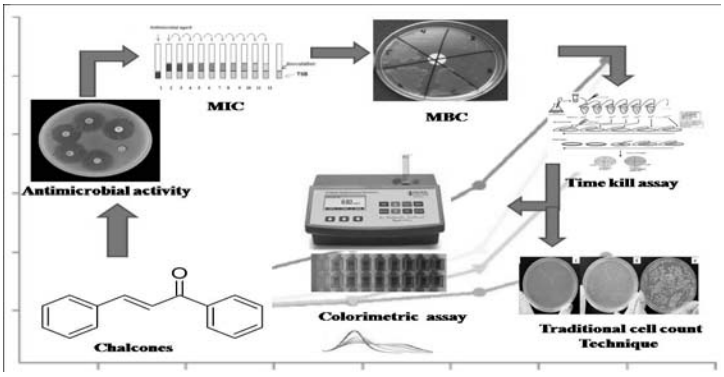
An attempt was made here to synthesize and screen the chalcones against certain Gram positive and Gram negative bacteria by adopting the modified colorimetric technique for Time-Killing Assay. A series of chalcones were synthesized from 1-(2-bromophenyl) ethanone and different substituted aromatic aldehydes with good yield and resolute on the basis of spectral data (FT/IR, ^{1}H NMR and ^{13}C NMR). The synthesized chalcones were screened for antibacterial activity against certain Gram positive and Gram negative bacteria. The pharmacokinetic profiles of chalcones were established by Time-Kill Assay, performed by colorimetry using resazurin dye as an indicator which colors only the viable cells. The ethoxy (I-4) and parahydroxy (I-5) derivatives were found to be more potent against Gram positive bacteria. The colorimetric Time-Kill Assay was found to be more convenient and less time consuming than traditional cell viable count.



INTRODUCTION

The rate of life-threatening infectious diseases has been increased day by day throughout the world which is mainly caused by multidrug-resistant organisms. Antibacterial are the agents which inhibit the survival and growth of microorganisms without any serious toxicity to the host. Resistance towards antibiotics becomes a major problem to treat and control the infectious diseases. Mainly because of the development of resistance by adapting themselves to withstand against the antimicrobial activity of the drug by mutations, membrane permeability, Bio-film formation and different drug effluxing mechanisms, their clinical utility was withdrawn from the market within a short period.

The emergence need of antibiotics is closely tied to the rapidity and raise of resistance developed by the organisms. The unbelievable ability of micro-organisms to develop resistance mechanisms for

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commonly used antibiotics has created a permanent need for the continued search for new active substances against pathogens.[1] The recent outbreak of research activities is to develop the drugs for multidrug resistant organisms. There are always some challengeable factors influencing the anti-microbial drug discovery such as the appearance of new diseases and requirement of various new screening techniques.

Nowadays, there are several analytical techniques available for the determination of a Time-Killing Assay in a manner more precise and faster than those previously more commonly used, which were much more time-consuming and tedious, laborious colony counting procedures. Here, an attempt has been made to test the synthesized chalcones using resazurin as the cell viability reagent by using the colorimetric technique.

Chalcone is a generic term given to compounds bearing the 1, 3-diphenyl-2- propen-1-one framework and which belong to the flavonoid family.

Chalcones show various activities such as anti-inflammatory, antifungal,[2] antioxidant,[3] antimalarial,[4] antitumor[5] and antibacterial[6] activities. Based on this view of biological importance of chalcones and in a trust of our investigation on antimicrobial agents, attempts were made here to synthesize and screen the chalcones against certain Gram positive and Gram negative bacteria by adopting the modified colorimetric technique for Time-Killing Assay.

MATERIALS AND METHODS

The chemicals were procured from Sigma Aldrich, Bangalore (for synthesis) which was used without any purification; Assay medium from Hi Media, Mumbai (for antibacterial studies), and all other chemicals and solvents used were analytical grade. Melting point was conceded out in an open capillary tube by using a melting point apparatus LAB INDIA MR-VIS Scientific. The purity of the synthesized compounds was confirmed by TLC (pre-coated) SiO_2 gel aluminum plates (E-MERCK) and visualized in iodine chamber. IR spectra were recorded on JASCO FT/IR-140 spectrophotometer. UV spectra were recorded on JASCO V-530 UV/VIS spectrophotometer.

TEST BACTERIAL STRAINS

All the strains were procured from National Chemical Laboratory, Pune, preserved and periodically subcultured. The 24h old culture was used for the assay.

SYNTHESIS OF CHALCONES

The compounds were synthesized by the reported method.[7] A solution of 22 g of sodium hydroxide in 200 mL of water and 100 mL of ethanol was taken in a 500 mL bolt-head flask provided with a mechanical stirrer and immersed in an ice bath. Freshly prepared 0.02 mole of 1-(2-bromophenyl) ethanone was added followed by addition of 0.01 mole of substituted benzalaldehyde and then it was stirred. The temperature of the mixture was maintained at 25°C and stirred vigorously until the mixture became thick and further stirring was no longer effectual. Then the stirrer was detached and the reaction blend was kept in refrigerator overnight. The product was filtered with the help of suction on a Buchner funnel, washed with cold water until the washings are neutral to litmus and then washed with 20 mL of ice-cold ethanol. The crude chalcones obtained, after dried in the air, were recrystallized using rectified spirit. The purity of the chalcones was established by single spot on the TLC plate. The solvent system used was toluene: methanol: ethyl acetate (6:3:1).

SPECTRAL DATA OF CHALCONES

2-bromophenyl-3-(4-hydroxy-3-methoxyphenyl) prop-2-en-1-one **(I-1)**: Yield: 80%. mp*:* 93-96°C. IR *(ν_{max}, cm^{-1}):*1676.8(C = O), 1633.41(C = C), 1021.12(C-O), 567.934(C-Br). U.V *[λ max*, *DMF* (*Dimethyl Formamide)]:* 301.^{1}H NMR*: (DMF-d_6*, *δ*, *ppm):* 3.75 (t, 3H), 6.68 (s, 1H), 6.74 (s, 1H), 7.2 (s, 1H), 7.47 (s, 1H), 7.56 (s, 1H), 7.59 (s, 1H), 7.65 (d, 2H), 7.94 (s 1H). ^{13}C NMR *($CDCl_3$) δ:* 55.43, 112.76, 114.68, 118.3, 122.32, 123.65, 127.5, 127.60, 127.82, 131.57, 132.65, 140.65, 142.60, 146.83, 148.56 186.43.

2-bromophenyl-3-[4-(dimethyl amino)phenyl] prop-2-en-1-one **(I-2)**: Yield: 78%. mp: 151-152°C. IR *(ν_{max}, cm^{-1}):*1676.8(C = C), 1333.53(C-N), 568.898(C-Br), 1676.8(C = O).*U.V (λ max*, *DMF):* 338. ^{1}H NMR: *(DMF-d_6*, *δ*, *ppm):*2.85 (m, 6H), 6.47 (s, 1H), 6.92 (d, 2H), 7.44 (d, 2H), 7.53 (t, 3H), 7.65 (s, 1H), 7.80 (s, 1H). ^{13}C NMR (*$CDCl_3$*) *δ*: 40.30, 40.30, 111.87, 111.87, 119.3, 122.06, 122.97, 126.85, 128.52, 128.52, 127.65, 131.52, 132.82, 141.75, 143.26, 150.43, 184.02.

2-bromophenyl-3-(3-nitrophenyl) prop-2-en-1-one **(I-3)**: Yield: 82%. mp: 120-121°C. IR *(ν_{max}, cm^{-1})*: 1636.3(-C = O), 1350.89(C-NO_2), 549.613(C-Br). U.V *(λ max, DMF)*: 295.^{1}H NMR: *(DMF-d_6, δ, ppm)*: 6.72 (s, 1H), 7.41(s, 1H), 7.54 (s, 1H), 7.16 (t, 3H), 7.89 (s, 1H), 7.98 (s, 1H), 8.31 (s, 1H), 8.62 (s,1H).^{13}C NMR (*$CDCl_3$*) δ: 115.34, 116.54, 118.23, 122.76, 126.54, 127.89, 128.34, 128.45, 128.90, 129.56, 132.78, 140.56, 141.76, 143.54, 184.91.

2-bromophenyl-3-(3-ethoxy-4-hydroxyphenyl) prop-2-en-1-one **(I-4)**: Yield: 80%. mp: 137-139°C. IR *(ν_{max}, cm^{-1}):* 1677.77(-C = O), 1021.12(C-O), 618.074(C-Br).U.V *(λ max, DMF):* 294.^{1}H NMR: *(DMF-d_6, δ, ppm)*: 1.25(t, 3H), 4.07 (d, 2H), 6.65 (s, 1H), 6.74 (s, 1H), 7.37 (s, 1H), 7.45 (s, 1H), 7.53 (s, 1H), 7.60 (s, 1H), 7.64 (s, 2H), 7.93 (s, 1H). ^{13}C NMR (*$CDCl_3$*) δ: 63.55, 108.76, 114.69, 118.7, 121.66, 122.17, 127.53, 127.82, 129.78, 131.76, 132.87, 139.98, 141.76, 144.67, 153.56, 185.12.

2-bromophenyl-3-(4-hydroxyphenyl) prop-2-en-1-one **(I-5)** Yield: 76%. mp: 134-137°C. *IR (ν_{max}, cm^{-1})*: 1676.8 (-C = O), 1013.41(C-O), 568.79 (C-Br).U.V *(λ max, DMF)*: 291.^{1}H NMR: *(DMF-d_6, δ, ppm):*6.63 (s, 1H), 6.87 (d, 2H), 7.42 (s, 1H), 7.54 (q, 4H), 7.60 (s, 1H), 7.89 (s, 1H). ^{13}C NMR (*$CDCl_3$*) δ: 116.04, 116.06, 119.3, 123.76, 126.39, 127.66, 128.5, 129.09, 129.18, 129.57, 134.87, 140.76, 142.59, 157.83, 184.67.

ANTIBACTERIAL ACTIVITY

The newly synthesized chalcones were screened for antibacterial activity using *Staphylococcus aureus* NCIM 2079, *Bacillus subtilis* NCIM 2063, *Escherichia coli* NCIM 2918, *Pseudomonas aeruginosa* NCIM 2036.

INOCULUM PREPARATION

The inocula of the testing bacteria were prepared by using the colony suspension technique.[8] Colonies taken from 24 h old cultures grown on nutrient agar slant were used to make suspensions of the test organisms in saline solution to furnish an optical density of approximately 0.1 at 600 nm.

The suspension was diluted to 1:100 by transferring 0.1 mL of the bacterial suspension to 9.9 mL of sterile nutrient broth before being used.

ANTIMICROBIAL ASSAY BY AGAR DIFFUSION METHOD

Well plate technique[9] by swabbing Mueller-Hinton Agar (MHA) plates with the inocula was prepared. By using a sterile 6 mm cork-borer, wells were bored into the agar media and filled with 50 µL of different concentrations of the synthesized chalcones and blank (DMF). Sufficient care was taken to avoid spillage of drug solutions onto the surface of the agar media. The culture plates were allowed to diffuse on the laminar air flow bench for 1 h and were incubated for 18–24 h at 37°C. After 24 h of incubation, the plates were examined for zone of inhibition.[10] The diameters of the inhibition zones were measured by using the CLSI (Clinical and Laboratory Standards Institute) zone diameter interpretative standards[11] and the procedure was duplicated.

MACRO BROTH DILUTION FOR DETERMINING MINIMUM INHIBITORY CONCENTRATION (MIC)

The MICs of synthesized compounds against Gram positive and Gram negative strains were performed.[12-14] Different concentrations (3.90–2 000 µg/mL) of the synthesized compounds were prepared by serial dilutions in the Mueller Hinton broth medium. Each tube was then inoculated with 100 µL of inocula. Two blank tubes of Mueller Hinton broth were run in parallel with and without bacterial inoculation and the same were used to confirm the sterility and growth. The tubes were incubated aerobically at 37°C for 24 h and examined for the MICs after the incubation period. The turbidity of the tubes was analyzed in ascending order of the drug concentrations and the first tube which shows no visible growth was considered as the MIC. The macro broth tubes of MIC were further used to determine Minimum Bactericidal Concentrations.

MINIMUM BACTERICIDAL CONCENTRATIONS (MBC)

100 µL of thoroughly mixed aliquot samples from all the macroscopically clear tubes including the first turbid tube of the series were sampled and placed on an antibiotic free nutrient agar plate by using single down streak from the center of the plate.[15] The samples were allowed to absorb into

the media until the plate surface appeared dry (after 30 min). The aliquot was then spread over the plate by making a lawn of the bacterial culture with a sterile cotton swab.[16-19] The controls for sterility and growth were also run in parallel. The inoculated plates were incubated for 24 h at 37°C. After the incubation, the plates were assessed for the growth of organisms and the plate in which the lowest concentrations of the synthesized compound that did not produce any bacterial growth was considered as the MBC value for the synthesized compounds.[20]

RATE KILL ASSAY

The Time-Kill Assay for the rate of killing bacteria by the synthesized compounds was carried out by using a modified plating technique.[21,22] The synthesized compounds at ½ MIC, MIC and 2 × MIC concentrations were added to 20 mL of Mueller Hinton broth in McCartney bottles along with 1 mL of inoculum (10^7cfu/mL or 10^7 Colony-Forming Unit/milliliter) and two control bottles were also run in parallel. The bottles were incubated at 37°C on an orbital shaker at 120 rpm. Two one mL samples at the given time intervals of 0, 4, 8 16 and 24 h were taken and one sample was processed for viable count and the other for colorimetric assay.

VIABLE COUNT DETERMINATION

Viable counts were determined by preparing 10-fold serial dilutions of the initial 1 mL sample in phosphate buffered saline and the growth control solutions is to equal a resultant concentration of 5×10^5 cfu/mL. An aliquot of 25 µL from each of the resultant dilutions was added to the Mueller Hinton Agar (MHA) plates[23] which were then incubated for 24 h at 37°C. Colonies were counted as discussed by Pankuch.[24] Time killing curves were constructed by plotting $\log_{10}$ cfu/mL against time over 24 h for the antibacterial effect of synthesized compounds. Adequate care was taken to run controls in parallel.

COLORIMETRIC ASSAY

A semi-quantitative measure of Time-Kill Assay was determined by using resazurin-reduction assay[1]. Resazurin is a reagent that functions as an indicator by utilizing the reducing capacity of only living cells. Upon contact with the cells, blue resazurin is reduced by cellular reducing agents during metabolism and cell growth to form pink resorufin. As both forms are photometrically discernible it is possible to assess the cell viability.[25] To each sample resazurin reagent (0.015 % w/v) was added and it was measured the absorbance at $\lambda_1 = 570$ nm and $\lambda_2 = 600$ nm after further incubation (2 h, 37°C).

RESULTS AND DISCUSSION

SPECTRAL CHARACTERIZATION

The strategy adopted to obtain the chalcones was depicted in Scheme 1. The structures of all the synthesized compounds were resolute on the basis of spectral data (FT/IR, ^{1}H NMR and ^{13}C NMR) and were in agreement with the proposed structures. The ^{1}H NMR data of the synthesized compounds showed signals between 7.24 and 7.91 δ ppm for aromatic protons of substituted phenyl ring. The signals between 6.45 and 6.67 δ ppm confirm the presence of olefinic (HC = CH) group. The ^{1}H NMR of I-1 compounds revealed the signal at 3.75 δ ppm (triplet) which confirms the presence of methoxy group. The presence of dimethyl group in compound I-2 was confirmed by the appearance of signal at 2.85 δ ppm as multilet. For compound I-4 the signals at 1.25 δ ppm (triplet) and 4.07 δ ppm (doublet) confirm the presence of ethyl group and the signal at 6.87 δ ppm confirms the presence of Aromatic hydroxyl group in compound I-5.

^{13}C NMR spectra of the synthesized compounds have shown characteristic signals between 122.76 and 153.56 δ ppm which confirms the presence of aromatic carbon atoms. The signals at 184.02 to 186.43 δ ppm confirm the presence of carbonyl carbon atom. The presence of olefinic HC = CH carbon atom was confirmed by the signals at 111.69 to 119.3 δ ppm. The signals at 56.15 δ ppm and 63.55 δ ppm reveal the presence of methoxy group in I-1 and I-4. The signals at 40.03 δ ppm confirm the presence of dimethyl group in I-2. The hydroxyl group attached to carbon atom in I-5 was confirmed by the signals at 157.83 δppm.

Furthermore, in IR spectra, the bands at 1676.8 to 1636.3 cm^{-1} and 567.934 to 618.07 cm^{-1} confirm the presence of C = O and C-Br groups. Moreover, another characteristic band appeared at 1333.53 cm^{-1} corresponding to the presence of C–N groups. The presence of NO_2 group attached to aryl ring for compound I-3 was confirmed by the characteristic band at 1350 cm^{-1} respectively.

The UV absorption spectra of the synthesized chalcones were recorded over the range of 200 and 400 nm by using DMF as a solvent at concentration (10^{-5}). The absorption peaks (λ_{max}) in UV spectra of compounds have shown signals at 291, 294, 295, 301 and 338 nm respectively for the compounds I–1 to I–5. It is also hereby confirmed that π–π* occur in the compounds due to the presence of unsaturated bonds. This π–π* is responsible for biologic activities of the compounds.[25]

ANTIBACTERIAL STUDIES

The compounds were evaluated for antibacterial activity, while the synthesized chalcones exhibited significant antibacterial activity against both Gram positive and Gram negative bacteria at different concentrations as shown in Table 1.

The agar diffusion assay indicated that the bacterial growth was inhibited by the synthesized chalcones to produce concentration dependent inhibition zones. Relatively the compounds I-4 and I-5 showed good antibacterial activity. The bacterial inhibition zones produced by the synthesized chalcones ranged between 13 and 19 mm. The degree of the antibacterial activity of the synthesized compounds was assayed by the serial two fold macro dilution technique to determine the MIC. The MIC values ranged between 31.25 µg/mL and 500µg/mL for both Gram positive and Gram negative bacteria (Table 2). The MBC values were 2–4 folds higher than that of MIC values of the synthesized compounds (Table 3). A good correlation was observed in the results obtained from agar diffusion and macro broth dilution assays. The compounds I-4 and I-5 showed a good antibacterial activity against Gram positive bacteria than the Gram negative bacteria.

Table 1

Zone of inhibition(mm) of antibacterial activity of newly synthesized compounds

Compound code	Diameter of Zone of inhibition(mm)							
	Gram positive bacteria				Gram negative bacteria(µg/mL)			
	SA **500 (µg/mL)**	***SA*** **1000 (µg/mL)**	***BS*** **500 (µg/mL)**	***BS*** **1000 (µg/mL)**	***EC*** **500 (µg/mL)**	***EC*** **1000 (µg/mL)**	***PA*** **500 (µg/mL)**	***PA*** **1000 (µg/mL)**
I-1	14±0.02	16±0.03	13±0.02	14±0.04	14±0.03	16±0.04	14±0.01	16±0.05
I-2	17±0.03	18±0.04	16±0.02	18±0.04	14±0.03	15±0.03	14±0.04	16±0.03
I-3	14±0.04	16±0.03	15±0.05	17±0.03	13±0.02	15±0.02	12±0.04	14±0.03
I-4	17±0.02	20±0.03	16±0.02	20±0.02	16±0.03	19±0.04	16±0.02	18±0.02
I-5	16±0.04	19±0.02	17±0.03	20±0.03	15±0.03	17±0.03	16±0.03	18±0.04
Control	-	-	-	-	-	-	-	-
Ofloxacin (5µg)	34±0.04		32±0.05		32±0.04		30±0.03	

Data are means (± sd) from duplicate in the experiments.
(-) indicates no zone of inhibition.
***SA*=*Staphylococcus aureus* NCIM 2079, *BS*= *Bacillus subtilis* NCIM 2063, *EC*= *Escherichia coli* NCIM 2911, *PA*= *Pseudomonas aeruginosa* NCIM 2036.**

Table 2

Minimal inhibitory concentration of newly synthesized compounds

Minimal Inhibitory Concentration (µg/mL)				
Compound Codes	**Gram positive bacteria**		**Gram negative bacteria**	
	SA	***BS***	***ES***	***PA***
I-1	500	500	125	250
I-2	125	250	250	500
I-3	500	500	250	500
I-4	31.25	62.5	62.5	125
I-5	62.5	125	125	125

Table 3

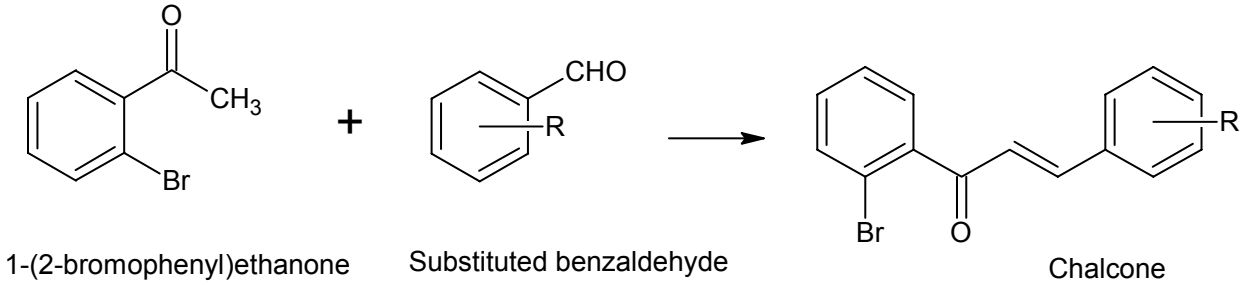
Minimal bactericidal concentration of newly synthesized compounds

Minimal bactericidal Concentration (µg/mL)				
Compound Codes	**Gram positive bacteria**		**Gram negative bacteria**	
	SA	***BS***	***ES***	***PA***
I-1	1000	1000	500	500
I-2	500	500	500	1000
I-3	1000	1000	500	1000
I-4	125	250	125	250
I-5	250	250	250	250

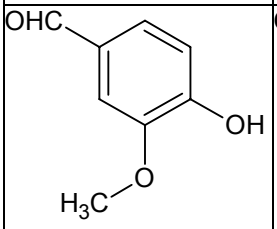
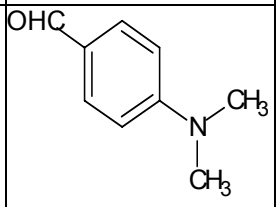
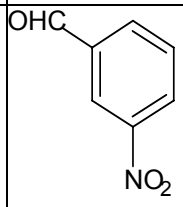
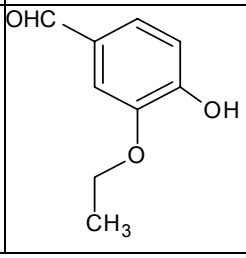
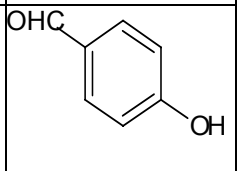
***SA*=*Staphylococcus aureus NCIM 2079*, BS=*Bacillus subtilis NCIM 2063*,*EC*= *Escherichia coli* NCIM *2911*, PA= *Pseudomonas aeruginosa*NCIM 2036.**

TIME KILL ASSAY

Time-Kill Assay was performed by both viable count and the resazurin reduction assay by using colorimetry, for all the compounds synthesized, against both Gram positive and Gram negative bacteria. The data of Time-Kill Assay by viable count expressed in terms of the $\log_{10}$ cfu/mL and resazurin reduction assay by colorimetry were expressed in terms of absorbance against time in hours. The results obtained were found to be well correlated in the both techniques. Average log reduction of viable cell count in Time-Kill Assay and average change in absorbance of compounds I-4 and I-5, against Gram positive and Gram negative organisms at various time intervals and at various concentrations ½ × MIC, 1 × MIC, 2 × MIC, were depicted graphically in Figs. 1, 2. For other compounds the graphical data were given as additional information. At the concentration of ½ × MIC, all the compounds have shown a slight increase in the respective values for both Gram positive and Gram negative bacteria. But the rate of growth was less when compared to the growth of control. Initially at 1h time interval at the concentration of 1 × MIC of all the compounds there is no reduction in the respective values. But by the time increases to 2, 4, 8, 16 and 24 h time intervals there is a decline in the count and absorbance. These values indicate that initially there is no inhibition or suppression of organism growth but as time increases there was a decline in growth. At the concentration of 2 × MIC there was a sharp decline in the viable count and absorbance of both Gram positive and Gram negative bacteria even from first sample (1h time interval) onwards. Based on the pharmacokinetic profiles, the tested compounds can be divided into three categories, *viz.* bactericidal with time dependent activity; bactericidal with concentration-dependent activity; and others with bacterio-static activity. By comparing the obtained pharmacokinetic profiles, all the synthesized compounds were categorized as bactericidal with concentration-dependent activity as the activities vary with the concentrations of the compounds *viz.* ½ × MIC, 1 × MIC and 2 × MIC. The study also had given a picture that colorimetric Time-Kill Assay provides enough data within a short time and also found to be less tedious than compared to viable count technique.



Scheme 1 – Synthesis of Chalcone.

Substituted benzaldehyde				
I-1	I-2	I-3	I-4	I-5
				

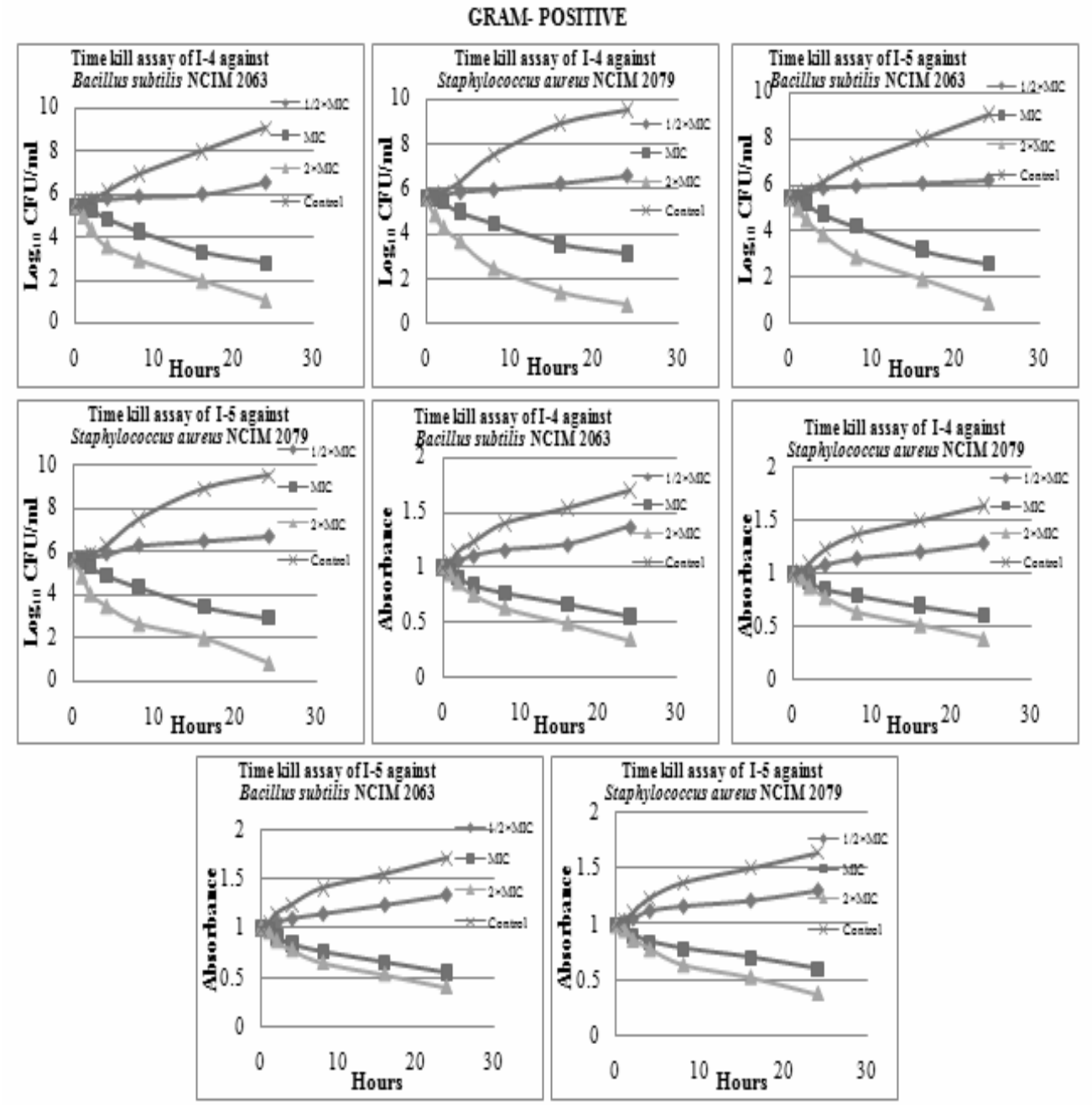


Fig. 1

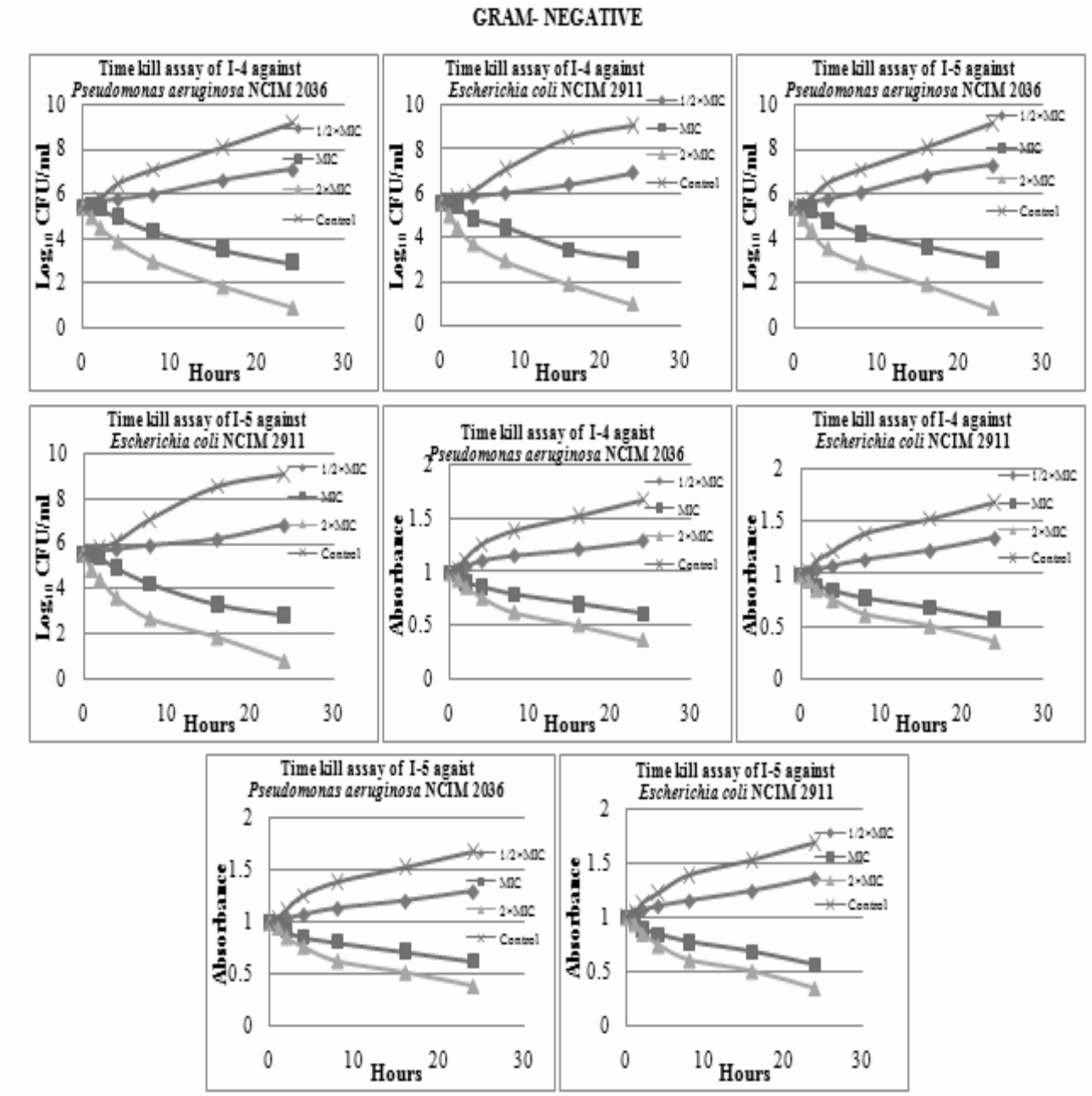


Fig. 2

CONCLUSION

It may be concluded that the newly synthesized compounds with nitro, hydroxyl, methoxy, ethoxy and dimethyl groups as substituent on phenyl ring of chalcones have shown significant antibacterial activity against both Gram positive and Gram negative bacteria. The compounds I-4 and I-5 showed comparatively good activity against Gram positive bacterial strains. All the compounds were found to exhibit better inhibitory action at a low concentrations against Gram positive and Gram negative organisms. The colorimetric Time-Kill Assay provides more complete data within a short time and less tedious than compared to viable count. Colorimetric method is likely to be developed as a standard technique to be used in modern microbiological studies. The present investigation exposed that chalcone derivatives can act as a potential lead for the development of new antibacterial agents and also increase the utility of the colorimetric method for Time-Kill Assay with resazurin as an indicator for staining the viable organisms for the antimicrobial studies.

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