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SCREENING OF ANTIMICROBIAL PROPERTIES OF PHENOL SCHIFF BASES

N. A. Ghanwate[*1], A. W. Raut [2] and N. N. Vidhale [3]

Department of Microbiology [1], Sant Gadge Baba Amravati University, Amravati - 444602, Maharashtra, India.
Department of Chemistry [2], Department of Microbiology [3], Shri Shivaji Science College, Amravati - 444603, Maharashtra, India.

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Correspondence to Author:
Dr. Niraj A Ghanwate

Assistant Professor,
Department of Microbiology, Sant Gadge Baba Amravati University, Amravati - 444602, Maharashtra, India.

E-mail: nirajghanwate@gmail.com

ABSTRACT: In their fight for supremacy and survival, microorganisms have been developing and devising various methods to inactivate the action of antibiotics. The numbers of drug resistant microorganisms with reduced susceptibility to various antibiotics are increasing. Therefore, new infection-fighting strategies are required to control microbial infections. The interaction between potent chemicals and living system contribute to the understanding of life processes and provide effective methods for treatment, prevention and diagnosis of many diseases. Phenol Schiff bases like chalcone imines and flavone imines were screened for their antimicrobial activity against a wide range of pathogenic bacteria and fungi. The *in vitro* activity of the compounds was evaluated by the Kirby-Bauer disc diffusion method and the MIC was determined for a few selected compounds. Most of the compounds showed significant antimicrobial activity. The compounds were found toxic to bacteria like *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *S. typhimurium*, *Shigella flexneri* and *Proteus* species. *Pseudomonas aeruginosa* was found to be resistant to most of the compounds. It was noted that all the compounds were active against fungi like *Cryptococcus neoformans*, *Candida albicans*, *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Mucor* and *Rhizopus* but inactive towards *Aspergillus niger*, *Asp. flavus* and *Asp. fumigatus*. It was interesting to note that the compounds were more fungi toxic than antibacterial. Flavone imines showed more antimicrobial activity than chalcone imines.

INTRODUCTION: Schiff bases have attracted much more attention due to their biochemical and biological activities [1] such as antiviral [2], anticancer [3, 4], antimicrobial[5-8] and antibacterial[9,10]. Anticancer Schiff bases have been synthesized by condensation of aniline with substituted benzaldehyde [11]. Schiff bases like chalcone imines have been reported to exhibit antimicrobial properties [12, 13].

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Preparation of 2'-hydroxy substituted [14-16] chalcone imine has been reported. Synthesis of 2'-hydroxy-3'- Nitro- 5- chloro- 4- substituted- N-(substituted phenyl) chalcone imine have been reported [17]. 2-Hydroxy chalcones and 2-hydroxy substituted chalcone are prepared by known methods [18].

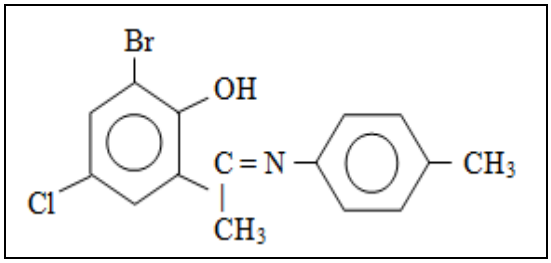
Chalcone condenses with substituted aniline in ethanol in presence of 2, 3 drops of concentrated H_2SO_4 to give chalcone imine. Chalcone imine reacts with DMSO-I_2-H_2SO_4 system to give flavone imine. DMSO-I_2 with or without H_2SO_4 reagent has been used for oxidative cyclization of 2-hydroxy chalcone to flavones and dehydrogenation to flavonoids [19]. Phenol Schiff bases with DMSO-I_2-

H_2SO_4 system give N-phenyl benzisoxazolines [20-22]. Synthesis of 8-nitro-5-chloro-4-substituted-N-(substituted phenyl) flavone imine has been reported [23].

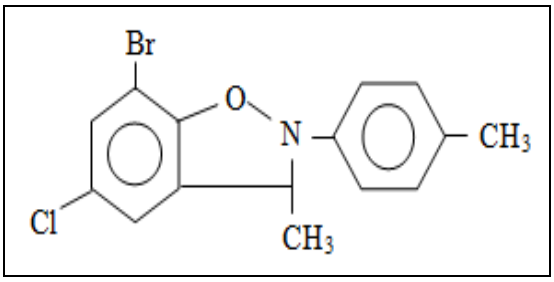
The synthesized Schiff bases were screened for their antimicrobial activity against bacteria like *E. coli*, *Klebsiella pneumoniae*, *Salmonella typhi*, *Salmonella typhimurium*, *Shigella flexneri*, *Proteus* sp., *Proteus mirabilis*, *Proteus vulgaris*, *Pseudomonas aeruginosa*, *Aerobacter aerogenes*, *Staphylococus aureus* and *Bacillus* sp. [24, 25] and fungal isolates like *Cryptococus neoformans*, *Candida albicans*, *Trichophyton mentagrophytes*, *Microsporum gypseum*, *Mucor*, *Rhizopus*, *Aspergillus niger*, *Aspergillus flavus* and *Aspergillus fumigates* [26] by disc diffusion method [27] by dissolving the compounds in methanol [28]. Those Schiff bases with good antimicrobial activity were further evaluated for their minimum inhibitory concentration.

MATERIAL AND METHODS: 2'-Hydroxy-3'-bromo-5'-chloro-4-methoxy chalcone condenses with p-toludene in ethanol in presence of 2,3 drops of conc. H_2SO_4 gives 2'hydroxy-3'-bromo-5'-chloro-4-methoxy-N-(para tolyl) chalcone imine m.p. 146 °C, yield 72%.

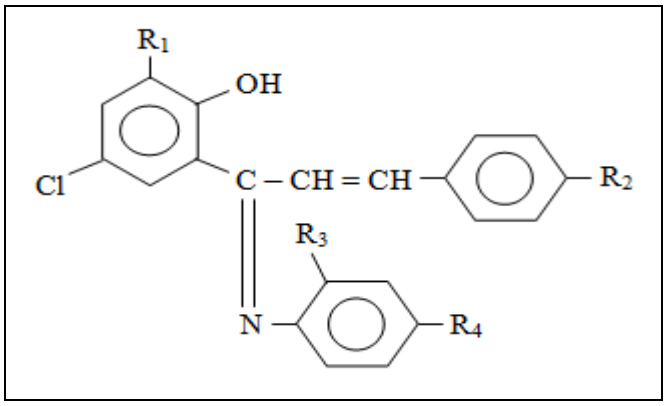
The following compounds were synthesized by known methods [29-31].



IIA: 2- HYDROXY- 3- BROMO- 5- CHLORO- 1- (α-PARAMETHYL PHENYL-IMINO) ETHYL BENZENE



IIB: 7- BROMO- 5- CHLORO- 3- METHYL- 2- (PARA TOLYL) BENZISOXAZOLINE

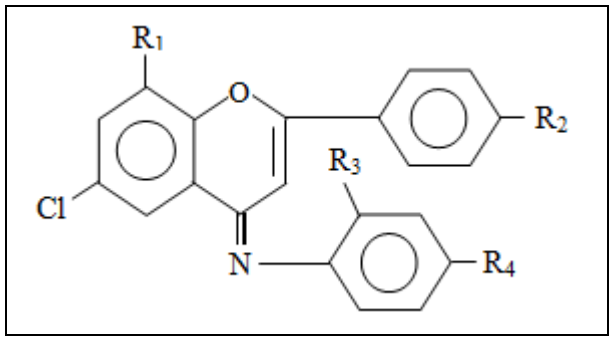


CHALCONE IMINE

TABLE 1: THE FOLLOWING CHALCONE IMINES WERE SYNTHESIZED

Sr. No.	Compound No.	R_1	R_2	R_3	R_4	m.p. (°C)	Yield (%)
3.	IIId	Br	H	NO_2	H	115	75
4.	IIIf	H	H	H	H	120	72
5.	IIIg	H	H	H	CH_3	330	70
6.	IIIh	H	$-OCH_3$	H	CH_3	330	72
7.	IIIi	H	$-OCH_3$	$-NO_2$	H	205	70
8.	IIIj	H	H	$-NO_2$	H	115	74

Substituted chalcone imines were dissolved in DMSO (40ml) and conc. H_2SO_4, 2, 3 drops was added. The mixture was refluxed for 10 min. It was then cooled and little catalytic amount of iodine was added. The reaction mixture was again heated for 1 hour in water bath then cooled and diluted with cold water. The resulting solid mass was treated with water 10% Sodium thiosulphate solution to remove iodine and again by water and crystallized from alcohol acetic acid mixture to get flavone imine.



FLAVONE IMINES

TABLE 2: THE FOLLOWING FLAVONE IMINES WERE SYNTHESIZED

Sr. No.	Compound No.	R_1	R_2	R_3	R_4	m.p. (°C)	Yield (%)
9.	IVa	Br	$-OCH_3$	H	$-CH_3$	312	80
10.	IVb	Br	H	H	$-CH_3$	285	82
11.	IVc	Br	$-OCH_3$	$-NO_2$	H	127	80
12.	IVd	Br	H	H	H	147	80
13.	IVe	H	$-OCH_3$		$-CH_3$	330	81
14.	IVf	H	$-OCH_3$	$-NO_2$	H	130	82

The compounds were tested *in vitro* for their antimicrobial activity by disc diffusion method.

Bacterial and Fungal Culture: Twelve pure bacterial cultures and eight pure fungal isolates were selected for the study. The cultures were procured from the Department of Microbiology, Government Medical College, Nagpur. The culture were confirmed by the conventional techniques [24-26] and maintained by sub culturing on semisolid nutrient agar and stored at 4-8 °C. NCTC strains of *S. aureus, E. coli* and *Pseudomonas aeruginosa* were used as control strains.

TABLE 3: MICROORGANISMS

Gram Negative	Gram Positive	Yeast	Yeast like Fungi	Moulds
Escherichia coli NCTC 10418	*Staphylococcus aureus* NCTC 6751	*Cryptococcus neoformans* ATCC 14116	*Candida albicans* ATCC 10231	*Trichophyton mentagrophytes* ATCC 9533
Salmonella typhi	*Bacillus* species	-	-	*Microsporum gypseum*
Salmonella thyphimurium ATCC 51812	-	-	-	Mucor
Shigella flexneri ATCC 9199	-	-	-	*Aspergillus niger* ATCC 16404
Klebsiella pneumoniae ATCC 10031	-	-	-	*Aspergillus flavus*
Aerobacter aerogenes	-	-	-	*Aspergillus fumigatus*
Proteus mirabilis ATCC 12453	-	-	-	-
Proteus vulgaris ATCC 33420	-	-	-	-
Proteus spp.	-	-	-	-
Pseudomonas NCTC 10662	-	-	-	-

Antibiotic Susceptibility Testing: The compounds were tested *in vitro* for their antimicrobial activity by Kirby Bauer disc diffusion method [21].

Preparation of Discs: Wet discs were used 6.25 mm diameter discs were punched from No. 1 Whatman filter paper and dispensed in batches of 100 in screw-capped bottles and sterilized by dry heat at 140 °C for 60 minutes. The selected compounds were dissolved in methanol [22] and their solutions were prepared such that 1 ml contains 100 times the amount of antibiotic required in the disc. 1ml of this solution was added to each bottle of 100 discs and as the whole of this volume was absorbed, it was assumed that each disc will contain approximately 0.01ml of the solution.

Culture Medium: The culture medium used was the HI-media India (make) Nutrient Agar for bacterial cultures and Sabouraud Dextrose Agar for fungal isolates.

Preparation of Inoculum: A small loopful (about 0.01 ml) of an overnight broth culture (about 10g bacteria/ml) was added to 5ml sterile isotonic saline to a concentration of 10^5-10^6 bacteria per ml.

Test Procedure: The culture plates were dried in the incubator with the lid ajar until its surface was free from moisture. 2ml of the inoculum suspension was transferred to the plate with a sterile Pasteur pipette and the plate was rotated to wet the whole of its surface. The excess fluid was removed with the pipette and the plates were dried in the incubator for 30 minutes. The compound discs were applied (2cm apart) to the surface of the plate with sterile fine pointed forceps and gently pressed to ensure full contact with the medium.

Immediately the plates were transferred to the incubator and incubated at 37 °C for 24 hours for bacteria and at 28 °C for 72 hours for fungi.

Reading Results: After incubation degree of sensitivity to compounds was determined by measuring the clear visible areas of growth free zones (zones of inhibition) produced by diffusion of compounds in the media. Zones of inhibition measured (including 6.25 mm of disc diameter) by calipers were reported in mm as shown in **Table 4** and **5**.

TABLE 4: BACTERIA SHOWING SENSITIVITY TO THE COMPOUNDS (SENSITIVITY MEASURED AS ZONE OF INHIBITION IN mm)

Compd.	*Escherichia coli*	*Klebisellan pneumoniae*	*Salmonella typhi*	*Salmonella typhimurium*	*Shigella flexneri*	*Proteus* spp.	*Proteus merabilis*	*Proteus vulgaris*	*Pseudomonas aeruginosa*	*Aerobacter aerogenes*	*Staphylococcus aureus*	*Bacillus* spp.
IIa	9	13	9	8	9	-	11	11	9	9	8	8
IIb	9	10	-	9	8	-	-	10	-	8	12	11
IIId	-	-	8	-	-	-	9	13	-	8	9	9
IIIf	8	9	13	16	13	-	8	12	11	8	-	8
IIIg	8	8	-	10	11	9	10	8	-	9	-	9
IIIh	11	11	10	8	9	10	8	9	-	9	-	9
IIIi	9	9	-	13	18	8	9	8	9	15	-	9
IIIj	11	11	8	8	-	8	8	8	-	11	-	10
IVa	-	11	9	9	12	-	9	9	9	8	14	10
IVb	11	14	9	9	16	9	9	11	-	9	11	8
IVc	9	10	9	8	9	10	12	9	10	11	14	11
IVd	-	-	-	-	-	-	-	8	-	8	8	8
IVe	11	-	13	9	-	10	8	9	-	11	16	11
IVf	11	-	12	13	9	-	-	8	-	10	14	10

TABLE 5: FUNGI SHOWING SENSITIVITY TO THE COMPOUNDS (SENSITIVITY MEASURED IN mm)

Compd.	*Cryptococcus neoformans*	*Candida albicans*	*Trichophyton mentagrophytes*	*Microsporum gypseum*	*Mucor*	*Rhizopus*	*Aspergillus niger*	*Aspergillus flavus*	*Aspergillus fumigatus*
IIa	8	-	11	12	9	8	13	13	13
IIb	8	9	10	8	9	10	-	-	-
IIId	12	-	-	-	9	11	-	-	-
IIIf	11	9	12	12	13	11	-	-	-
IIIg	10	10	9	-	-	-	-	-	-
IIIh	-	9	12	10	11	10	-	-	12
IIIi	9	10	14	14	12	11	-	-	-
IIIj	12	9	14	11	12	12	-	-	12
IVa	15	13	10	11	11	12	8	-	-
IVb	10	8	12	12	11	13	8	-	12
IVc	20	13	22	20	15	18	11	20	25
IVd	-	-	-	-	-	-	-	-	-
IVe	12	9	15	13	10	10	-	-	15
IVf	23	15	20	18	19	15	20	20	25

Evaluation of Minimum Inhibitory Concentration (MIC) of a Few Selected Compounds: After screening of 14 compounds, 3 compounds with good antimicrobial activity were selected for evaluation of their MIC values. The following compounds were selected-

- **IIa:** 2-Hydroxy-3-bromo- 5- chloro-1- (α-para methyl phenyl imino) ethyl benzene.
- **IIIi:** 2-Hydroxy-5-chloro-4-methoxy- N- (ortho nitro phenyl) chalcone imine.
- **IVf:** 6-Chloro-4-methoxy-4-ortho nitro phenyl imino flavone.

The MIC was determined by disc diffusion method [21] against the different bacteria stated in **Table 1**.

Disc with Different Concentration of Compounds: Stock solutions of the selected 3 compounds were prepared in methanol, each with a concentration of 10,000 µg/ml. Different concentrations were prepared from the stock solution. One ml from each concentration was added to 100 sterile discs. The final concentration per disc was 100, 80, 60, 40, 20 and 10µg. Culture medium, inoculum, test procedure and reading of results were carried out as described above.

Determination of MIC: The MIC of the compounds for a bacterial isolate was estimated from measurement of the zone of inhibition in the disc test by reference to a standard graph which related the MIC to the zone diameter for the control

strain of known degree of sensitivity. Discs containing different amounts of the compound were tested under carefully standardized conditions against the following control strains.

1. *Escherichia coli* NCTC 10418.
2. *Pseudomonas aeruginosa* NCTC 10662.
3. *Staphylococcus aureus* NCTC 6571.

Initially the minimum concentration of *E. coli* NCTC 10418, *Pseudomonas aeruginosa* NCTC 10662 and *Staphylococcus aureus* NCTC 6571 was determined towards the 3 selected compounds (IIa, IIIi and IVf). MIC of the compounds was determined by incorporating the compound in the nutrient agar in the concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140µg/ml. The inoculum of the control strain was prepared as in case of disc diffusion method and 1-2ml of the inoculum was applied on the agar surface. The plates were incubated at 37 °C for 18-24 hours and results read. The lowest concentration of the compound at which there was no visible growth was taken as the minimum inhibitory concentration.

Later the test micro-organisms were tested under the same conditions. The concentration of compound required for the inhibition of growth (MIC) was calculated from the measurement of the diameter of the zone of inhibition by reference to the standard graph.

The degree of sensitivity of the test bacteria was calculated as follows.

$$\text{MIC of Test bacteria} = \frac{\text{Amount of antibiotic per disc required to inhibit the test bacterium at a given size of zone}}{\text{Amount of antibiotic per disc required to inhibit the control strain at the same size of zone}} \times \text{MIC of control strain}$$

TABLE 6: MIC OF IIa, IIIi AND IVf AGAINST THE CONTROL STRAINS (µg/ml)

Compound	*E. coli* NCTC 10418	*Pseudomonas aeruginosa* NCTC 10662	*Staphylococcus aureus* NCTC 6571
IIa	50	120	80
IIIi	30	50	70
IVf	30	40	60

TABLE 7: MIC (µg/ml) OF COMPOUNDS IIa, IIIi AND IVf FOR DIFFERENT TEST BACTERIA

S. no	Test Bacteria	IIa	IIIi	IVf
1.	*Escherichia coli*	16.66	34.60	90
2.	*Klebsiella pneumoniae*	62.5	45.75	90
3.	*Aerobacter aerogenes*	25	75	75
4.	*Proteus vulgaris*	31.25	30	75
5.	*Proteus mirabilis*	41.5	32	80
6.	*Proteus* spp.	-	45.75	-
7.	*Salmonella typhi*	81.66	R	45.75
8.	*Salmonella typhimurium*	27.77	45	34.60
9.	*Shigella flexneri*	22.70	35	112.50
10.	*Pseudomonas aeruginosa*	-	166.66	-
11.	*Staphylococcus aureus*	106	-	180
12.	*Bacillus* spp.	80	122.5	51.42

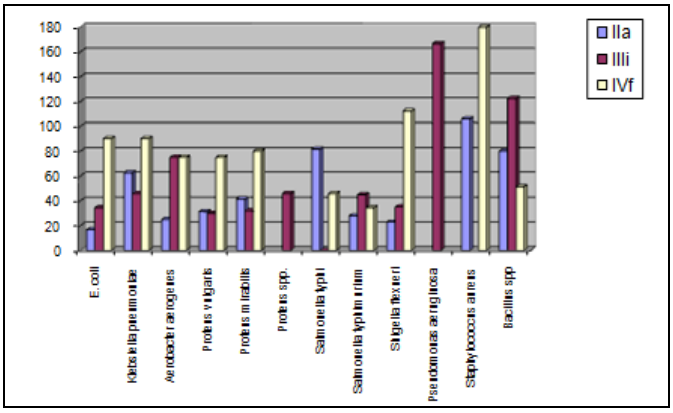


FIG. 1: MIC OF COMPOUNDS IIa, IIIiAND IVf FOR DIFFERENT TEST BACTERIA

DISCUSSION: From the results it is clear that flavone imines showed greater antimicrobial activity than chalcone imines. Antibacterial activity of chalcone imines is highest against *Salmonella typhimurium*, *Proteus vulgaris* and *Shigella flexneri*, moderate against *E. coli*, *Klebsiella*, *Proteus mirabilis* and *Aerobacter*. It is inactive against *S. aureus*. Synthesis and antibacterial activity of 2-hydroxy-5-bromo-N-(substituted phenyl) chalcone imine was also reported by Shubhangi *et al.*, against *S. aureus*, *E. coli*, *Pseudomonas aeruginosa* and *S. typhimurium* [32]. Synthesis, characterization and study of antimicrobial activity of 2- hydroxy 3-nitro-5-methyl-n-(substituted phenyl) chalcone imines was also reported by Borul and Agarkar in 2015 [33] and Xianwen *et al.*, in 2014 [13]. While the flavone imines are very active against *S. aureus*, *Bacillus* and *Aerobacter* though moderate to others.

The well-known fact that *Pseudomonas* and *S. aureus* show multiple drug resistance is also noted in this study. All fungi are sensitive to almost all the compound except the *Aspergillus* species which are found to be sensitive only to IVc and IVf. Here also the flavone imines were more active than the chalcone imines. Cytotoxic and antimicrobial activity of some synthetic flavones were studied and reported by Sohel *et al.*, against selected bacterial and fungal strain [34]. Antifungal activity of chalcone imines is highest against the dermatophytes and candida while flavone is more active against dermatophytes, mucor, Rhizopus and Aspergillus.

It was seen that the presence of -OCH_3 group invariably increased the antimicrobial activity of these compounds. It is also found that compounds containing bromo and nitro group together are more active than simple one.

CONCLUSION: All the compounds except III d and IV d were found to exhibit strong antibacterial and antifungal properties. It was also concluded that flavones imines (compounds IV a and IV f) were more active than chalcone imines. As compared to other compounds, II a, III I and IV f showed the highest antimicrobial activity.

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CONFLICT OF INTEREST: The authors have no conflict of interest.

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