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Comparison study of Antimicrobial activity with effect of DPPH for Antioxidant study of Synthesized Schiff bases of Mannich bases of Resacetophenone having variable Electronegative Atoms (:O:/:S:/:NH)

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Abstract

Three Schiff bases of Mannich bases of resacetophenone have been synthesized by using three variable electronegative atoms (X=O/S/NH) of urea/thiourea/guanidine and tested for antimicrobial activity and antioxidant screening by DPPH. It has been found that Compound-3a (X=O) showed maximum zone of inhibition in *E.coli* & *S.aureus* and minimum zone of inhibition in *B.subtilis*, Compound-3b (X=S) showed maximum zone of inhibition in *E.coli* in comparison with standard drug streptomycin. There is no activity found in case of Compound-3c (X=NH). Antimicrobial activity profile of three compounds is as follows:

Gm positive bacteria: **Compound-3a** *(S.aureus > B.subtilis)*

Gm negative bacteria: **Compound-3b** *(E.coli)* > **Compound-3a** *(E.coli)*

LogP values of three compounds were found to be X=O: 4.03, X=S: 4.63 and X=NH: 3.79. So, the partition coefficient is in this order: X=S: 4.63 > X=O: 4.03 > X=NH: 3.79. Partition coefficient of X=S is 4.63 which is greater than X=O (4.03) and X=NH (3.79) electronegativity of S is 2.58 which is in between O=3.44 and NH=3.04. Electronegative atoms O and S both have two lone pair of electrons but electronegativity for O: 3.44 > N: 3.04 > S: 2.58 and the % inhibition of oxidation by DPPH has been observed as NH (45.56) > O (37.76) > S > (36.5). The electronegativity of N is 3.04 so the NH compound (guanidine) showed maximum inhibition 45.46%, electronegativity of O is 3.44 so the O compound (urea) showed 37.76% inhibition and the electronegativity of S is 2.58 so the S compound (thiourea) showed 36.5% inhibition. The log P values of NH compound (guanidine) is 3.79 which is minimum but showed maximum inhibition 45.46%, log P value of O compound (urea) is 4.03 which showed in 37.76 % inhibition and log P value of S compound (thiourea) is 4.63 which showed 36.5% inhibition. Oxygen and Sulfur both have two lone pairs of electrons so the % inhibition values are under very narrow range O (37.76), S (36.5) but this slight variation is due to the difference in electronegativity: O: 3.44 and S: 2.58.

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Key words:

Schiff base, Mannich base, logP, IC_{50}, DPPH, Electronegativity, Antioxidant, Gm+ve bacteria, Gm-ve bacteria

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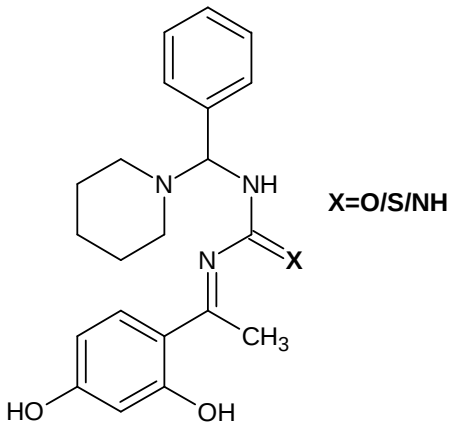
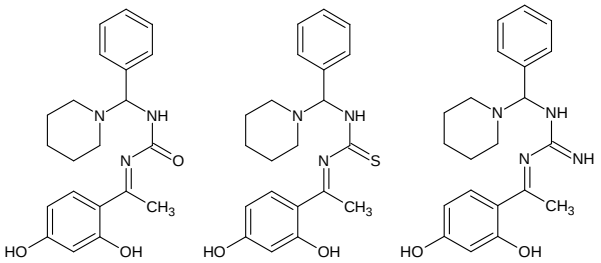
Introduction

The proposed planning has been designed to incorporate Schiff base and Mannich base together in single compound with variable electronegative atoms O/S/NH by using urea, thiourea and guanidine for **X**. All the three derivatives of resacetophenone have been tested for antimicrobial as well as antifungal activity with MIC and antioxidant property by logP values (Table-1). All the compounds were characterized by UV, IR, NMR and Mass spectrum analysis with N%.



Molecular skeleton



X=O: Urea X=S: Thiourea X=NH: Guanidine

Desired molecules

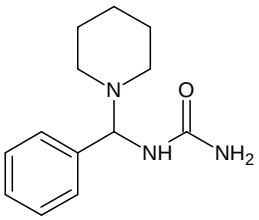
Synthesis of Mannich bases:

1 mole benzaldehyde was treated with 1 mole piperidine dissolved aqueous solution of 1 mole of urea/thiourea/guanidine and heated on water bath until the entire product solidified. It was placed in ice and filtered, washed with cold water to remove unreacted benzaldehyde and piperidine. It was then recrystallised with charcoal in aqueous ethanol.[1-3]

1a: 1-[phenyl (piperidin-1-yl)-methyl]-urea X=O: $C_{13}H_{19}N_3O$

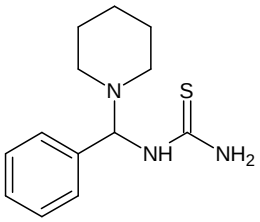
1b: 1-[phenyl (piperidin-1-yl)-methyl]-thiourea X=S: $C_{13}H_{19}N_3S$

1c: 1-[phenyl (piperidin-1-yl)-methyl]-guanidine XNH: $C_{13}H_{20}N_4$



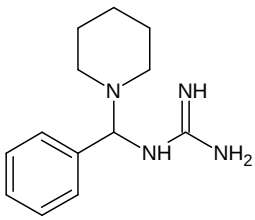
1-[phenyl(piperidin-1-yl)methyl]urea

1a



1-[phenyl(piperidin-1-yl)methyl]thiourea

1b



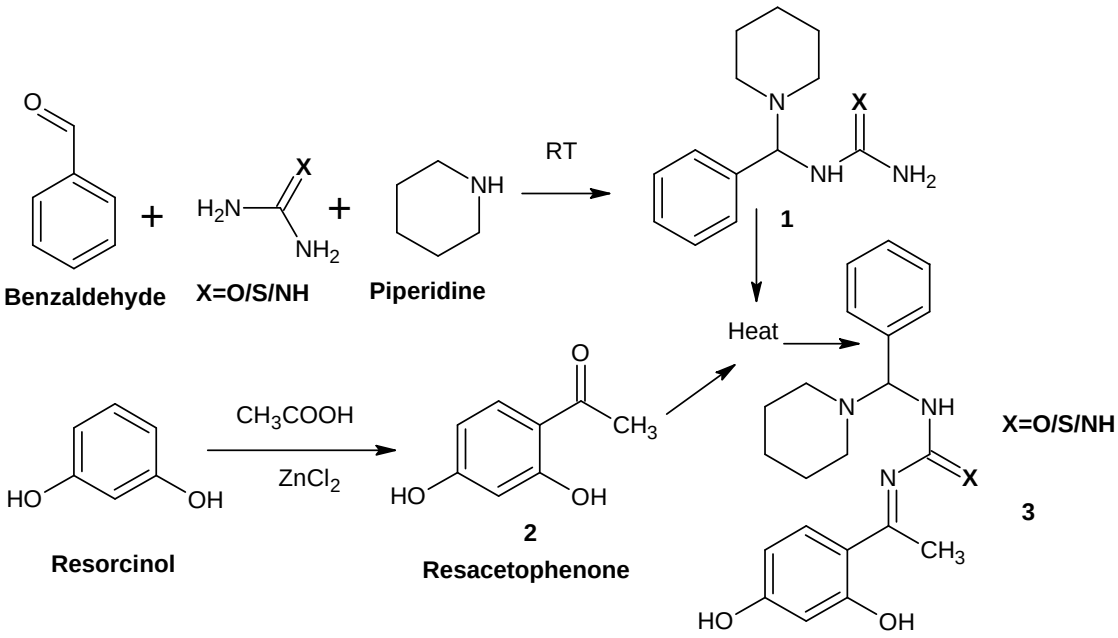
1-[phenyl(piperidin-1-yl)methyl]guanidine

1c

Synthesis of Resacetophenone: (Hoesch reaction)

1.2 moles of anhydrous zinc chloride was dissolved with the aid of heat in 2.7 moles of glacial acetic acid, which has been placed in a 1-l. beaker. To this hot mixture (about 140°C), 1 mole of resorcinol was added with constant stirring. The solution was heated on a sand bath until it just begins to boil (about 152°C). The flame was then removed and the reaction allowed completing itself at a temperature not in excess of 159°C. After standing on the sand bath without further heating for 20 minutes, the solution is diluted with a mixture of 250 ml. of concentrated hydrochloric acid and 250 ml. of water. The dark red solution was placed in an ice bath and cooled at 5°C. The resulting precipitate was collected on a filter and washed free from zinc salts with 1 l. of dilute (1:3) hydrochloric acid in 200-ml. portions. This orange-red product, after drying, weighs 104–110 g. and melts at 141–143°C. It was distilled under reduced

pressure and boils at 180–181°C at 10 mm. (147–152°C at 3–4 mm.). After most of the product has distilled, the temperature rises sharply and the operation was discontinued when the temperature reaches 190°C. The light-yellow distillate was removed from the receiver with hot ethanol and the ethanol was evaporated. This product weighs 100–106 g. It was further purified as follows: the substance was dissolved in 1.8 L of hot dilute (1:11) hydrochloric acid, filtered hot and cooled to 5°C. The crystals were removed by filtration, washed with two 200-ml. portions of ice water and dried. The yield of tan-colored resacetophenone, melting at 142–144°C, is 93–99 g. (61–65%).[4-6]



Scheme

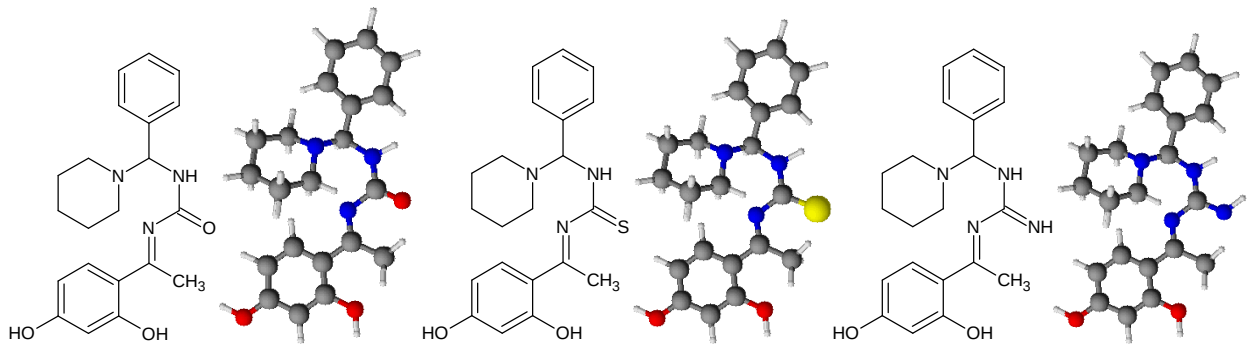
Synthesis of Schiff's bases:

1 mole of resacetophenone was heated with 1 mole of **3a/3b/3c** separately in rectified spirit on water bath until the entire product dissolved. It was continued for 30' and then placed in ice bath to get the crystals of Schiff's bases. The products were drained, washed with cold water and recrystallised with aqueous ethanol and charcoal to get the pure form.[7]

3a: X=O: $C_{21}H_{25}N_3O_3$ (367) N=11.44%

3b: X=S: $C_{21}H_{25}N_3O_2S$ (383) N=10.96%

3c: X=NH: $C_{21}H_{26}N_4O_2$ (366) N=15.29%



3a **3b** **3c**

Physicochemical Parameters:-

Compounds	% Yield	M.P.°C	Polarity	Solubility	Molecular Formula	Molecular Weight	N% Calculated	N% Found
3a	42.00	140-145	Non-polar	Ethanol, $CHCl_3$, CCl_4	$C_{21}H_{25}N_3O_3$	367	11.44	11.22
3b	40.52	155-160	Non-polar	Ethanol, $CHCl_3$, CCl_4	$C_{21}H_{25}N_3O_2S$	383	10.96	10.88
3c	50.00	190-194	Non-polar	Ethanol, $CHCl_3$, CCl_4	$C_{21}H_{26}N_4O_2$	366	15.29	15.23

Table-1

I.R. Spectras:-

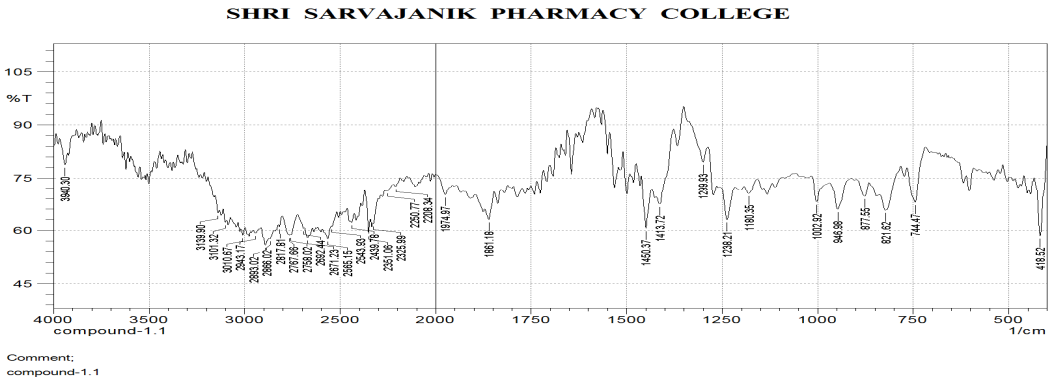
Phenolic –**OH** stretch 3900-3000 cm^{-1} very strong due to hydrogen bond formation and may obscures other band. In thus region particularly of –**NH** stretch. **C-H** stretching 3150-2700 cm^{-1} complex region due to many no. of hydrocarbons skeleton. **C=C** conjugated gives very stronger absorption around the region 1500-1400 cm^{-1}. **N-H** bend often obscured by stronger **C=C** absorption with polar substitution otherwise it observed at around 1500 cm^{-1}. Aromatic overtone region around 1800-1940 cm^{-1}. **N-H** stretch at 3305.78 cm^{-1} is quite clear seen on the top of the broad **O-H** stretch. **C=O** stretch not observed due to amide-amidol tautomerism so instead of it C-O bend observed at 1238.21 cm^{-1}. **C-H** bending observed at 950-700 cm^{-1}. **N-C=S** stretching at around 2250-1900 cm^{-1} here it is observed at 1907.47 cm^{-1}. **C-N** bending observed around 1300-1000 cm^{-1} and give complex region.

U.V. Spectras

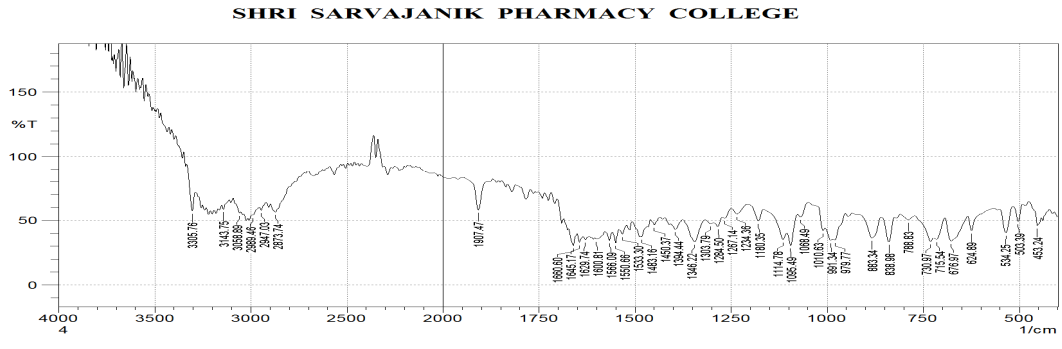
Compounds	λ_{max} (nm)
3a	290
3b	326
3c	280

Table-2

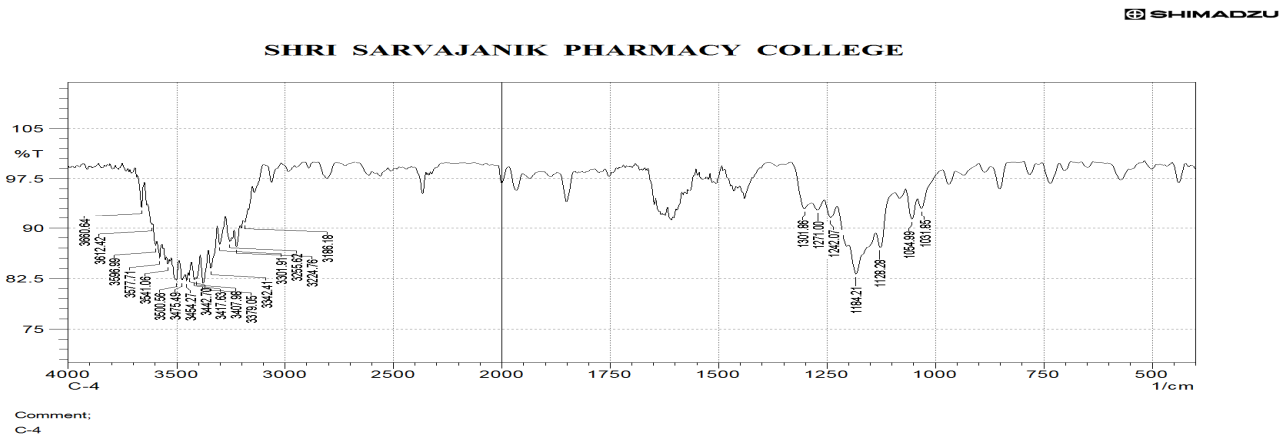
I.R. Spectras



Compound 3a: υ (KBr, cm^{-1}): 3940.30, 3139.90 (Phenolic –**OH**), 3150-2700 (-**C-H**, stretching), 1238.21 (-**C-O**), 1947.97, 1861.18 (-C_6H_5), 1450.37, 1413.72 (- **C-H**, bending), 744.47 (-C_6H_5, ortho), 821.62, 877.55 (-C_6H_5, para), Aromatic out of plane bending observed at 950-700 (-C_6H_5)



Compound 3b: υ (KBr, cm^{-1}): 3305.78 (-**N-H**, stretching), 3150-2700 (-**C-H**, stretching), 1600-1303 (**C-C**), 1500 (**C=C**), 1660 (**C=N**), 1300-1000 (**C-N**) , 1907.47 (**N-C=S**, stretching), 744.47 (-C_6H_5, ortho), 821.62, 877.55 (-C_6H_5, para), 950-700 (-C_6H_5, bending)



Compound 3c: υ (KBr, cm^{-1}): 3660.64, 3612.42, 3596.99, 3577.71, 3500.58 (**Phenolic –OH**, stretching), 3379.05 (-**N-H**, stretching), 3407.96, 3417.63, 3442.70, 3454.27 (**O-H**, stretching), 3186.18 (-C_6H_5, stretching), 1301.86-1031.85 (**C-N**) and **C-O**)

BIOLOGICAL SCREENING OF SYNTHESIZED COMPOUNDS (3a-3c)

Antimicrobial activity

The synthesized compounds (3a-3c) were tested for their antimicrobial activities by following Kirby-Bauer disc-diffusion method using nutrient agar medium against following pathogenic microorganisms:

Gram-negative bacteria: *Escherichia coli, Pseudomonas aeruginosa*

Gram-positive bacteria: *Staphylococcus aureus, Bacillus subtilis*

Name of Microorganism

Gram +Ve microorganisms

Staphylococcus aureus (MTCC No. 740)

Bacillus subtilis (MTCC No. 441)

Gram -Ve microorganisms

Escherichia coli (MTCC No. 443)

Preparation of medium:-

Nutrient agar	2%
Peptone	1%
Beef extract	1%
Sodium chloride	0.5%
Distilled water	up to 100ml

All the ingredients were weighed and added to water. This solution was heated on water bath for about one and half-hour till it became clear. This nutrient media was sterilized by autoclave.

Apparatus:-

All the apparatus like Petridishes, pipettes, glass rods, test-tubes etc. were properly wrapped with papers and sterilized in hot air oven.

Antimicrobial screening method

- All the Petri dishes were sterilized in oven at 160°C for 1 hour.
- Agar media, borer and test solutions were sterilized in autoclave at 121°C at 15psi.
- Molten sterile agar was poured in sterile petri dishes aseptically.
- The agar was allowed to cool and the bacterial suspension was poured into the petridishes aseptically.
- Placing the sterile filter paper discs in the agar plate and solution of the compounds was added by using pipette (0.1ml) in appropriate four quadrants of petridishes aseptically.

Agar plate disc diffusion method:

- The antibacterial activity has to be assayed by agar plate disc diffusion method at the concentration of 800µg/ disk.
- All the synthesized compounds have to be tested in vitro for their antibacterial activity against microorganisms such as *Staphylococcus aureus*, *Bacillus subtilis* (gram positive) *and Escherichia coli* (gram negative) strains.

- Each test compounds have to be dissolved in dimethylsulphoxide (DMSO) to get a concentration of 10 mg/mL.
- The disc (6 mm in diameter) has to be impregnated with 5µL of each test solution to get 50 µg/disc, air dried and placed on the agar medium, previously seeded with 0.2mL of broth culture of each organism for 18 hours.
- The plates have to be incubated at 37°C for 24 hours and the inhibition zones measured in mm.
- Discs impregnated with DMSO have to be used as a control and Ciprofloxacin discs as antibacterial reference standard.

Petridishes were incubated at 37°C for antimicrobial and 24°C for antifungal for 24 hrs and observed the zone of inhibition.

The microbiological assay is based upon a comparison of inhibition of growth of micro-organisms by measured concentrations of test compounds with that produced by known concentration of a standard antibiotic. Two methods generally employed are turbidometric (tube-dilution) method and filter paper method. In the turbidometric method inhibition of growth of microbial culture in a uniform dilution of antibiotic in a fluid medium is measured. It is compared with the synthesized compounds. Here the presence or absence of growth is measured. The cylinder plate method depends upon diffusion of antibiotic from a vertical cylinder through a solidified agar layer in a Petridis or plate to an extent such that growth of added micro-organisms is prevented entirely in a zone around the cylinder containing solution of the antibiotics. The cup-plate method is simple and measurement of inhibition of microorganisms is also easy. Here we have used this method for antibacterial screening of the test compounds.[8-10]

Minimum Inhibitory Concentration

- Minimum inhibitory concentration (MIC) of the test compounds have to be determined by agar streak dilution method.
- A stock solution of the synthesized compound [800µg/mL] in dimethylformamide has to be prepared and graded quantities of the test compounds have to be incorporated in specified quantity of molten sterile agar (nutrient agar for antibacterial activity and sabouraud dextrose agar medium for anti fungal activity)
- A specified quantity of the medium (40-50°C) containing the compound has to be poured into a petridish to give a depth of 3-4mm and allowed to solidify suspension of the microorganism have to be prepared to contain approximately 105Cfu/mL and applied to plates with serially diluted compounds in dimethylformamide to be tested and incubated at 37°C for 14h and 48h for bacteria and fungi respectively.

The MIC has to be considered to be lowest concentration of the test substance exhibiting no visible great of bacteria or fungi, on the plate.

Compounds 800 (µg/ml)	Strains	Zone of Inhibition (mm)	MIC µg/ml
3a	*Escherichia coli*	12	
	Staphylococcus aureus	12	
	Bacillus subtilis	07	
3b	*Escherichia coli*	17	700
	Staphylococcus aureus	00	700
	Bacillus subtilis	00	600
3c	*Escherichia coli*	00	
	Staphylococcus aureus	00	
	Bacillus subtilis	00	
Streptomycin	*Bacillus subtilis*	27	

Table-3

2,2-Diphenyl-1-picrylhydrazyl Radical Scavenging Capacity Assay.

DPPH is one of a few stable and commercially available organic nitrogen radicals and has a UV-vis absorption maximum at 515 nm. Upon reduction, the

solution color fades; the reaction progress is conveniently monitored by a spectrophotometer. The DPPH assay is typically run by the following procedure:

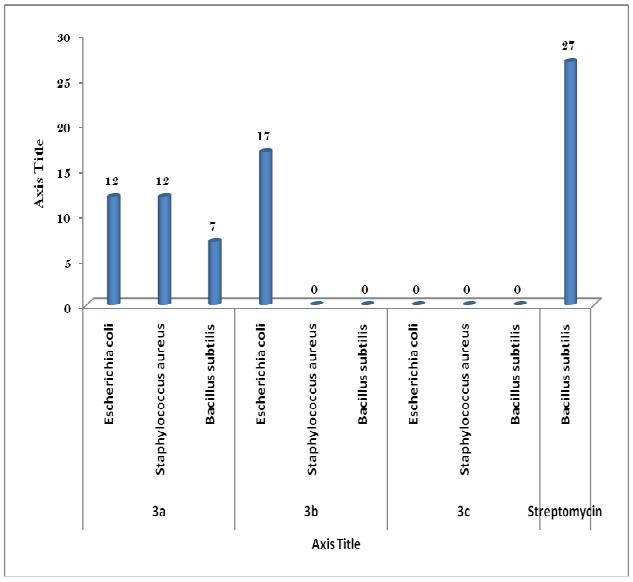
DPPH solution (3.9 mL, 25 mg/L) in methanol is mixed with sample solution (0.1 mL). The reaction progress absorbance of the mixture is monitored at 515 nm for 30 min or until the absorbance is stable. Upon reduction, the color of the solution fades. The percentage of the DPPH remaining is calculated as

$$\%DPPH_{rem} = 100 \times [DPPH]_{rem}/[DPPH]_{T=0}$$

%DPPHrem is proportional to the antioxidant concentrations, and the concentration that causes a decrease in the initial DPPH concentration by 50% is defined as EC_{50}. The time needed to reach the steady state with EC_{50} concentration is calculated from the kinetic curve and defined as TEC_{50}.

Sanchez-Moreno and co-workers classified the kinetic behaviour of the antioxidant compound as follows: < 5 min (rapid), 5-30 min (intermediate), and > 30 min (slow). They further proposed a parameter, called "antiradical efficiency (AE)", to express the antioxidant capacity of a certain antioxidant. AE is calculated as

$$AE = (1/EC_{50})TEC_{50}$$



Histogram of Antimicrobial screening

The DPPH assay is technically simple, but some disadvantages limit its applications. Besides the mechanistic difference from the hydrogen atom transfer reaction that normally occurs between antioxidants and peroxyl radicals, DPPH is a long-lived nitrogen radical, which bears no similarity to the highly reactive and transient peroxyl radicals involved in lipid peroxidation. Many antioxidants that react quickly with peroxyl radicals may react slowly or may even be inert to DPPH. This is evident from the TEC_{50} values ranging from 1.15 min (ascorbic acid) to 103 min (rutin). Consequently, the antioxidant capacity is not properly rated. The reaction kinetics between DPPH and antioxidants are not linear to DPPH concentrations. It is thus rather arbitrary to express antioxidant capacity using EC_{50}. Finally, it was reported that the reaction of DPPH with eugenol was reversible. This would result in falsely low readings for antioxidant capacity of samples containing eugenol and other phenols bearing a similar structure type (*o*-methoxyphenol).

The DPPH assay was believed to involve hydrogen atom transfer reaction, but a recent paper suggested otherwise. On the basis of the kinetic analysis of the reaction between phenols and DPPH, Foti and co-workers suggested that the reaction in fact behaves like an ET reaction. The authors found that the rate-determining step for this reaction consists of a fast electron transfer process from the phenoxide anions to DPPH. The hydrogen atom abstraction from the neutral ArOH by DPPH becomes a marginal reaction path, because it occurs very slowly in strong hydrogen-bond-accepting solvents, such as methanol and ethanol. In addition, the author found that adventitious acids or bases present in the solvent may dramatically influence the ionization equilibrium of phenols and cause a reduction or an enhancement, respectively, of the measured rate constants. This renders the DPPH assay much less chemically sound as a valid assay for antiradical activity of measurement.[11]

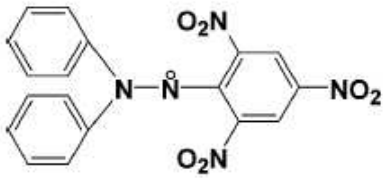
CALCULATION:

% DPPH radical-scavenging = (Absorbance of control – Absorbance of test sample) ÷ (Absorbance of control) × 100

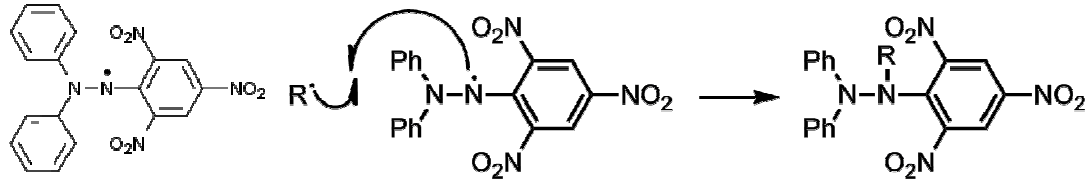
Abs. Cont.=absorbance of DPPH at 517 nm as a control after 30 min of reaction.

Purified sample 2 mg/ml in ethyl alcohol of synthesized compounds were taken for antioxidant activity with a standard BHA (Butylated Hydroxy Anisole) antioxidant for method suitability.

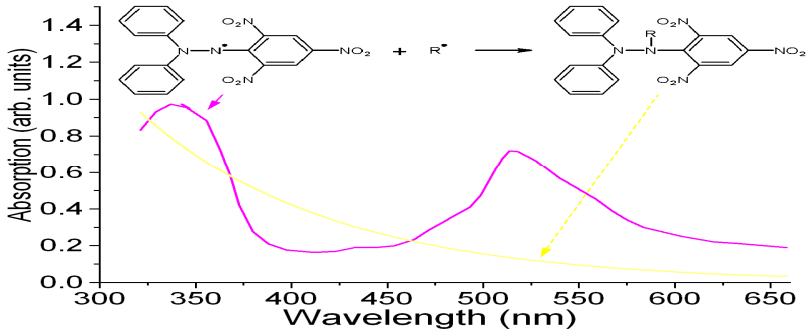
Decreased absorbance of the reaction mixture indicates stronger DPPH radical-scavenging activity.[12,13]



DPPH



IUPAC Name of DPPH: di (phenyl)-(2,4,6-trinitrophenyl)iminoazanium



IC_{50} values of Test Compounds

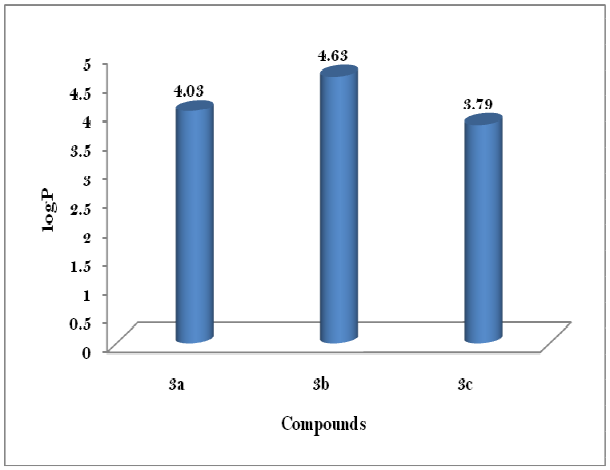
Compounds	Molecular Formula: Molecular weight	IC_{50} Value	logP
3a	$C_{21}H_{25}N_3O_3$: 367	1.33	4.03
3b	$C_{21}H_{25}N_3O_2S$: 383	1.48	4.63
3c	$C_{21}H_{26}N_4O_2$: 366	1.02	3.79

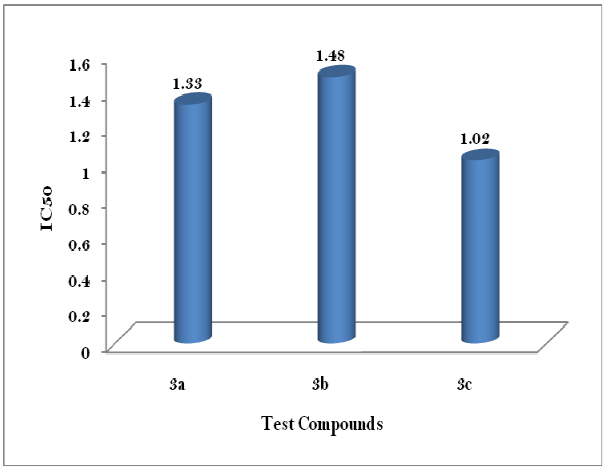
Table-4

% Inhibition of oxidation by three compounds

3a (X=O)		3b (X=S)		3c (X=NH)	
Conc. (mg/ml)	% Inhibition	Conc. (mg/ml)	% Inhibition	Conc. (mg/ml)	% Inhibition
0.1	13.76	0.1	16.36	0.1	14.22
0.3	20.48	0.3	19.72	0.3	22.62
0.5	26.6	0.5	27.06	0.5	28.89
0.7	30.42	0.7	28.74	0.7	37.3
0.9	37.76	0.9	36.5	0.9	45.56

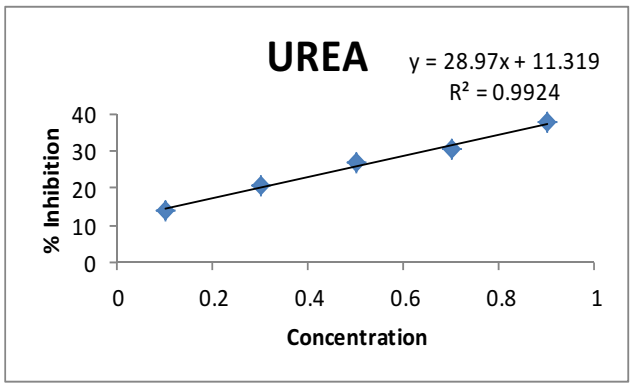
Table-5

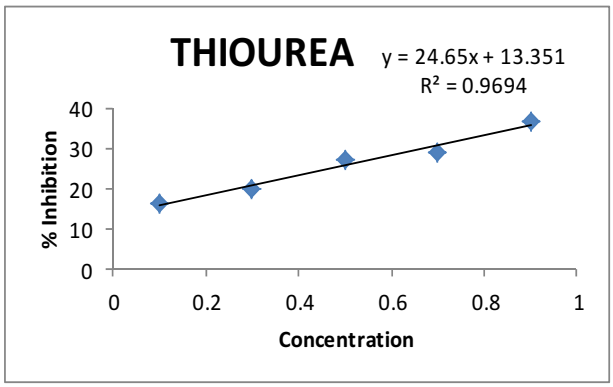


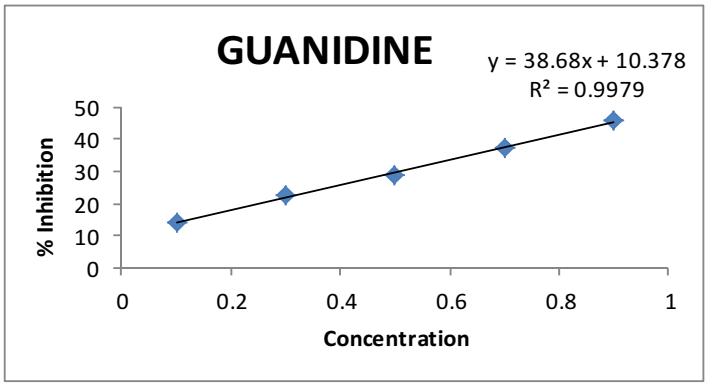


Histogram of log P and IC_{50} values

Linear plots of three test compounds







Linear Plot of 3a (urea), 3b (thiourea) and 3c (guanidine)

Conclusion: Three Schiff bases of Mannich bases of resacetophenone have been synthesized by using three variable electronegative atoms (X=O/S/NH) of urea/thiourea/guanidine and tested for antimicrobial activity and antioxidant screening by DPPH. It has been found that Compound-3a (X=O) showed maximum zone of inhibition in *E.coli* & *S.aureus* and minimum zone of inhibition in *B.subtilis*, Compound-3b (X=S) showed maximum zone of inhibition in *E.coli* in comparison with standard drug streptomycin. There is no activity found in case of Compound-3c (X=NH). Antimicrobial activity profile of three compounds is as follows:

Gm positive bacteria: **Compound-3a** *(S.aureus > B.subtilis)*

Gm negative bacteria: **Compound-3b** *(E.coli)* > **Compound-3a** *(E.coli)*

LogP values of three compounds were found to be X=O: 4.03, X=S: 4.63 and X=NH: 3.79. So, the partition coefficient is in this order: X=S: 4.63 > X=O: 4.03 > X=NH: 3.79. Partition coefficient of X=S is 4.63 which is greater than X=O (4.03) and X=NH (3.79) electronegativity of S is 2.58 which is in between O=3.44 and NH=3.04. Electronegative atoms O and S both have two lone pair of electrons but electronegativity for O: 3.44 > N: 3.04 > S: 2.58 and the % inhibition of oxidation by DPPH has been observed as NH (45.56) > O (37.76) > S > (36.5). The electronegativity of N is 3.04 so the NH compound (guanidine) showed maximum inhibition 45.46%, electronegativity of O is 3.44 so the O compound (urea) showed 37.76% inhibition and the electronegativity of S is 2.58 so the S compound (thiourea) showed 36.5% inhibition. The log P values of NH compound (guanidine) is 3.79 which is minimum but showed maximum inhibition 45.46%, log P value of O compound (urea) is 4.03 which showed in 37.76 % inhibition and log P value of S compound (thiourea) is 4.63 which showed 36.5% inhibition. Oxygen and Sulfur both have two lone pairs of electrons so the % inhibition values are under very narrow range O (37.76), S (36.5) but this slight variation is due to the difference in electronegativity: O: 3.44 and S: 2.58.

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