Research Article

Potent Enzyme Inhibitors: Synthesis and Spectral Analysis of Some New Schiff Bases Having Nucleus of Azomethine and Screened their Biological Evaluation

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ABSTRACT

Schiff bases **8-13**, have been synthesized by the condensation of 2-aminophenol **1** with aldehydes/kitones **2-7**. Their structures were characterized by spectroscopic (^{1}H-NMR, IR, EI-MS) data along with elemental analysis. The target compounds were biological screened out and showed good lipoxygenase, α-glucosidase inhibition and antioxidant activities, whereas compound **10** showed potent lipoxygenase inhibition activity. The compounds **8-10** and **13** were found as potent α-glucosidase inhibitors. The compounds **9** and **13** were found as potent antioxidant. All the compounds were found to have good activity against bacterial strains but had no significant activity against urease enzyme.

Keywords: Schiff bases, Synthesis, Molecular docking, Antioxidant, X-ray diffraction, Biological activities

INTRODUCTION

The study of antioxidants, enzyme inhibitors and antibacterials has become a vast research areas in the field of pharmaceutics and pharmaceutical chemistry, without which depreciation of human skin, body, disease, and death rate can't be controlled. The discovery of new and useful drugs for a variety of physiological conditions is the result of such type of research. The role of reactive oxygen species (ROS) is of prime consideration in various diseases because such species oxidize the human cell or body. The protection from such ROS species to human body is provided by antioxidants. The overview of literature showed that bacterial infection was the major cause for death of a large number of people. It has been found that harsh conditions can not affect a variety of species of *Bacillus, Staphylococcus, Salmonella and Pseudomonas* because such species can combat multiple environmental conditions which may lead to severe infections and fatal diseases. The morphological and biochemical modifications of bacteria and other microorganisms resulted in the resistance of such species against routinely used antibiotics. This fact has prompted a number of scientists to develop new drug candidates and antibiotic agents. The working principle of enzyme inhibitors involves blocking of the active sites of the enzymes to stop the activity of enzyme towards the substrates.

The Schiff bases have attracted the scientific community for designing new templates due to the fact that such compounds are antimalarial[1], anticonvulsant[2], anticancer[3], antibacterial[4], antifungal[5], anti-inflammatory[6], etc.

Moreover, they can be easily achieved through condensation of a carbonyl compound and an amine (Figure 1) with good yield. Medicinal value and importance of Schiff bases in various areas has developed our interest to synthesize such compounds[7].

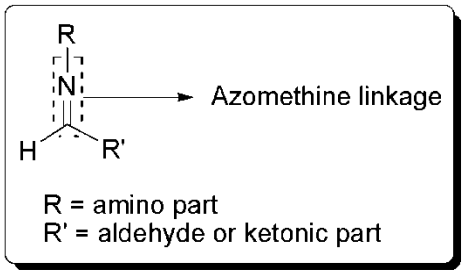

Figure 1: General representation of the structure of a Schiff base.

In this article, we report the synthesis and spectroscopic study of Schiff bases and evaluation of their antioxidant, lipoxygenase, urease inhibition, α-glucosidase inhibition and antibacterial activities.

MATERIALS AND METHODS

All the chemicals and solvents purchased from E. Merck and used as obtained. Thin layer chromatography (TLC) was performed on pre-coated silica gel G-25-UV254 plates (E. Merck), and detection was carried out at 254 and 366 nm. The IR spectra were recorded on Thermo Nicolet Avatar 320 FTIR spectrometer using KBr pellets. Melting points were measured on a Gallenkamp apparatus and are uncorrected. Elemental analyses were performed on Perkin Elmer 2400 Series II elemental analyzer. The ^{1}H NMR spectra were recorded on a Bruker AMX-400 spectrometer in DMSO-d_6. The chemical shifts

(*δ*) are given in ppm, relative to tetramethylsilane as an internal standard, and the scalar coupling constants (*J*) are reported in Hertz. The balance used for weighing was an electric Mettler Toledo balance, model AL 204. The recording of EI-MS spectra was conducted by electron impact mode on Finnigan MAT-112 spectrometer (Finnigan, Waltham, MA, USA) and *m/z* (%) of $[M]^+$ ions reported.

General Procedure for the Synthesis of Schiff Bases (8-13)

The addition of 3-4 drops of conc. H_2SO_4 was made to a mixture of 2-aminophenol **(1)** (0.01 mol in 50 mL EtOH) and aldehyde/ketone **(2-7)** (0.01 mol in 50 mL EtOH) and it was refluxed at 70 °C on water bath with stirring for 3 h. The resulting solution was concentrated by using rotary evaporator to one third of its volume and was cooled in an ice bath. The concentrated reaction mixture was kept at room temperature and solid products were obtained. The products were washed with methanol at ambient temperature and were recrystallized with methanol as well. The product thus recrystallized was dried under reduced pressure over anhydrous calcium chloride. The reaction was monitored by TLC after lapse of small intervals of time.

2-{[3-(4-Methoxyphenyl)allylidene]amino}phenol **(8):** Yield 70.21 %; IR (KBr, ν_{max} cm^{-1}): 1655 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 8.05 (1H, s, -HC=N-); EI-MS: *m/z* 253.2 $[M]^+$ (calcd. 253.1 for $C_{16}H_{15}NO_2$); Elemental analysis: Found (Calcd %); C: 75.83 (75.87), H: 5.99 (5.97), N: 5.57 (5.53).

2-[(2-Bromo-3-phenylallylidene)amino]phenol **(9):** Yield 75.31 %; IR (KBr, ν_{max} cm^{-1}): 1691 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 7.35 (1H, s, -HC=N-); EI-MS: *m/z* 301.0 $[M]^+$ (calcd. 301.0 for $C_{15}H_{12}BrNO$); Elemental analysis: Found (Calcd %); C: 59.68 (59.62), H: 4.12 (4.00), N: 4.69 (4.64).

2-Bromo-4-[(2-hydroxyphenylimino)methyl]phenol **(10):** Yield 83.91 %; IR (KBr, ν_{max} cm^{-1}): 1602 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 8.54 (1H, s, -HC=N-); EI-MS: *m/z* 290.9 $[M]^+$ (calcd. 290.9 for $C_{13}H_{10}BrNO_2$); Elemental analysis: Found (Calcd %); C: 53.56 (53.45), H: 3.61 (3.45), N: 4.83 (4.79).

2-[(Pyridin-3-ylmethylene)amino]phenol **(11):** Yield 67.32 %; IR (KBr, ν_{max} cm^{-1}): 1592 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 9.20 (1H, s, -HC=N-); EI-MS: *m/z* 198.0 $[M]^+$ (calcd. 198.0 for $C_{12}H_{10}N_2O$); Elemental analysis: Found (Calcd %); C: 72.85 (72.71), H: 5.19 (5.08), N: 14.34 (14.13).

4-(2-Hydroxyphenylimino)pentan-2-one **(12):** Yield 83.02 %; IR (KBr, ν_{max} cm^{-1}): 1595 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 7.15 (1H, dd, *J* = 7.6, 1.6 Hz, H-9), 7.01 (1H, ddd, *J* = 8.4, 8.0, 1.6 Hz, H-11), 6.89 (1H, dd, *J* = 8.0, 1.6 Hz, H-12), 6.78 (1H, ddd, *J* = 8.4, 7.6, 1.6 Hz, H-10), 5.19 (1H, s, H-3), 1.95 (6H, s, H-1, -6); EI-MS: *m/z* 191.0 $[M]^+$ (calcd. 191.0 for $C_{11}H_{13}NO_2$); Elemental analysis: Found (Calcd %); C: 69.14 (69.09), H: 6.93 (6.85), N: 7.34 (7.32).

2-{[4-(Diethylamino)benzylidene]amino}phenol **(13):** Yield 78.92 %; IR (KBr, ν_{max} cm^{-1}): 1589 (C=N); ^{1}H-NMR (DMSO-$_6$*d*, 400 MHz) δ: 8.46 (1H, s, -HC=N-); EI-MS: *m/z* 268.1 $[M]^+$ (calcd. 268.1 for $C_{17}H_{20}N_2O$); Elemental analysis: Found (Calcd %); C: 76.12 (76.09), H: 7.57 (7.51), N: 10.52 (10.44).

Pharmacological Assays

Antioxidant: DPPH Radical Scavenging Assay

The solution of DPPH (0.3 mM) was prepared in ethanol. 5 µL methanol solution of each sample of different concentration (5-500 µg) was mixed with 95 µL of DPPH solution in ethanol. The mixture was then dispersed in 96 well plate and incubated at 37 °C for 30 min, then absorbance was measured at 515 nm by microtitre plate reader (Spectramax plus 384 Molecular Device, U.S.A.). BHA is used as standard. The percent radical scavenging activity was determined in comparison to the methanol treated control with the following formula:

$$\text{DPPH scavenging effect (\%)} = \frac{\text{Absorbance of control (DMSO treated)} - \text{Absorbance of sample}}{\text{Absorbance of control}} \times 100$$

The IC_{50} value of the compounds was determined by monitoring the effect of different concentrations (1-1000 µM). The IC_{50} of the compounds were calculated using EZ-fit enzyme kinetic program (Pellera Scientific Inc. Amherst, U.S.A)[8].

Urease Inhibition Assay

The urease enzyme solution was prepared by taking 0.125 units in each well in phosphate buffer ($K_2HPO_4.3H_2O$, 1 mM EDTA and 0.01 M $LiCl_2$). Each well was filled with 80 µL of 0.05 M potassium phosphate buffer (pH 8.2), 10 µL of the test compound (concentration range 5 - 500 µM), contents were mixed and incubated for 15 min at 30 °C. 40 µL of substrate solution (urea) (50 mM) was added in each well for initiating reaction. Then, 70 µL alkaline reagent (0.5 % NaOH and 0.1 % active NaOCl) and 40 µL of phenol reagent (1% phenol and 0.005 % w/v sodium nitroprusside) were introduced to each well.

The reaction mixture containing well plates were incubated for 50 minutes and absorbance was recorded at 630 nm. IC_{50} values were determined by monitoring the effect of increasing concentrations of test compounds on extent of inhibition[8].

Lipoxygenase Inhibition Assay

All the chemicals including linoleic acid and lipoxygenase (EC 1.13.11.12) purchased from Sigma (St. Louis, Missouri, USA). 160 µL of 100 mM sodium phosphate buffer (pH 8.0) and 10 µL of test compound solution in methanol (of concentrations 5-500 µM) was added in each well. 20 µL of lipoxygenase (LOX) solution (enzyme 130 units per well) was added, mixed and incubated for 10 min at 25 °C. The reaction was then initiated by the addition of 10 µL

substrate solution (linoleic acid, 0.5 mM, 0.12 % w/v tween-20 in ratio of 1:2) in each well. The absorption changed with the formation of (9*Z*,11*E*)-13*S*)-13-hydroperoxyo-ctadeca-9,11-dienoate and was measured after 15 min at 234 nm. Baicalein was used as standard and IC_{50} values were determined by EZ-fit enzyme kinetic program (Pellera Scientific Inc. Amherst, U.S.A).

α-Glucosidase Inhibition Assay

The inhibitory activity of α-glucosidase was determined by modification of the previously reported method[9]. The inhibition assay was performed in 96 well microplate in a total volume of 100 µL. Standard solutions of the inhibitors were prepared in methanol. α-Glucosidase (from *Saccharomyces cerevisiae*) and *p*-nitrophenyl α-d-glucopyranoside (pNPG) as substrate were prepared in 0.07 M phosphate buffer (pH 6.8). The assay mixture was initially comprised of inhibitor solution (10 µL), buffer (70 µL) and 0.25 unit/mL enzyme solution (10 µL). This mixture was pre-incubated at 37 C for 5 min. After pre-incubation, *p*-nitrophenyl glucopyranoside (pNPG) (10 µL) was added as a substrate at 1 mM end concentration start the enzymatic reaction. The reaction mixture was incubated at 37 °C for 30 min and enzymatic reaction was stopped by adding 80 µL of 0.2 M Na_2CO_3. Negative control contained 10 µL of methanol instead of inhibitor. Acarbose was used as a positive control. The α-glucosidase activity was determined by measuring the amount of *p*-nitrophenol released from pNPG at 405 nm. The % inhibition was calculated by following equation:

$$\% \text{ Inhibition} = \left[1 - \left(\frac{\text{Absorbance of sample}}{\text{Absorbance of control}}\right)\right] x\ 100$$

IC_{50} values were calculated using non-linear curve fitting program PRISM 5.0 (GraphPad, San Diego, California, USA). The IC_{50} value was defined as the concentration of α-glucosidase inhibitor that inhibited 50% of α-glucosidase activity.

Antibacterial Assay

Antibacterial activities of compounds **8-13** was carried out against Gram-positive and Gram-negative bacteria viz. *B. subtilis, S. aureus, E. coli, S. typhi* and *P. aeruginosa* bacteria by modified agar well diffusion method using Mueller Hinton agar medium[10]. Each compound (200 mg) was dissolved in 10 ml 99.9 % dimethyl sulfoxide (DMSO) to get the concentration of 20 mg/ml. Test organism were grown individually in tryptic Soya broth for overnight and subsequently mixed with physiological saline until turbidity standard 10^8 Colony Forming Unit (CFU) per ml was achieved. Molton Mueller Hinton agar medium was seeded for individual organism with 10 ml of prepared inoculums (inoculum size was 10^8 cells/ml as per McFarland standard) and after proper homogenization, it was poured into 20×100 mm petri dishes. After solidification, required numbers of wells were made in the seeded plates with help of a sterile crock-borer (8.0 mm). The test compound (100 µl) was introduced into respective well. Positive control (gentamicin 0.3%) and negative control (DMSO) was also applied in each plate then all the plates were incubated at 37 ˚C for 24 h. Antibacterial activity was determined by measuring the diameter of the zone inhibition.

RESULTS AND DISCUSSION

Chemistry

The aldehydes/ketone **2-7** were condensed with 2-aminophenol **1** to achieve Schiff bases **8-13** with as demonstrated in Figure 2.

The Schiff bases **8-13** were highly stable in air which gives a clue about the purity of compounds. The completion of reaction for Schiff bases **8-13** was estimated by a direct comparison of reactants and reaction mixtures spot on TLC.

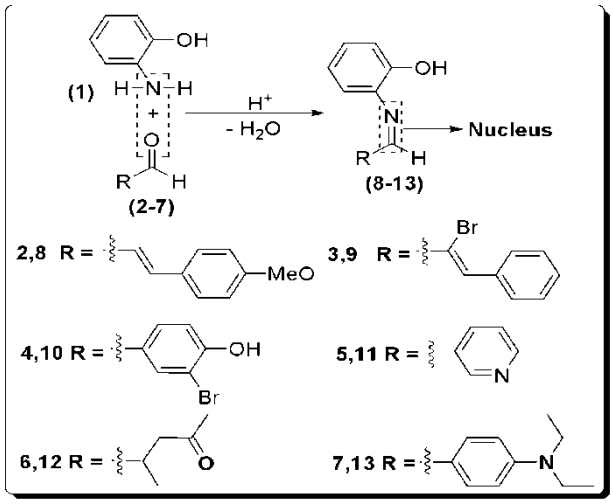

Figure 2: Synthesis of Schiff bases **(8-13)**.

Schiff Bases **(8-13)** *Characterization*

The data for elemental analyses of Schiff bases **8-13** was in complete harmony with the structure of respective Schiff base. In addition to that the confirmation of structures of Schiff bases **8-13** was made by IR, ^{1}H-NMR and EI-Mass spectrometry. In IR spectrum of schiff bases, the bands found in the range of 1691-1589 cm^{-1} reflected the presence of azomethine linkage (-HC=N-) linkage in the Schiff bases **8-13** and this reflection was further strengthened by the bands at 2800-2700 cm^{-1} due to aldehydic moieties.

The presence of peaks at 9.20-7.35 ppm respectively in ^{1}H-NMR of schiff bases **8-13**, showed the presence of azomethine group in the synthesized compounds which is a clear indication of the formation of Schiff base.

The molecular ion peaks $[M]^+$ for the Schiff bases **8-13** were observed in the EI-MS spectra of respective schiff bases which is a supportive evidence for successful synthesis. Moreover, the elemental analyses data for the schiff bases **8-13** was in complete agreement with their respective molecular masses.

Pharmacological Activities

Antioxidant: DPPH Radical Scavenging Activity

The normal metabolism involves the production of reactive oxygen species (ROS) which is considered to take part in human aging significantly *via* etiology and pathophysiology. Moreover, such species are the major cause of various diseases like cancer, Alzheimer's disease, coronary heart disease, atherosclerosis, cataracts, inflammation, and neurodegenerative disorders. Antioxidants are certain compounds which protect human body from oxidation by reacting with active oxygen. The most commonly used antioxidants in daily routine include butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT). The well diffusion methodology was employed by using DPPH for determining antioxidant activity of schiff bases **8-13**. Among synthesized compounds **9** and **13** showed potent antioxidant activity while compound **10** was found active to a significant level. The compounds **8** and **11** showed even moderate activity as compared with standard but **12** was found to have non-significant activity (Table 1).

Urease Inhibition Activity

The protein urease (EC 3.5.1.5) is found in yeast, bacteria, higher plants, and in *Helicobacter pylori* as well. Urease is also produced in many gastrointestinal pathogens. This enzyme contains nickel and is involved in catalyzing conversion of urea to ammonia and carbamate which ultimately decomposes to carbonic acid and ammonia, leading to an increase in pH level. The problems of gastric ulceration, pyelonephritis, urinary stone formation, and other dysfunctions are also associated with this enzyme. The synthesized compounds **8-13** were found to have non-significant activity against this enzyme (Table 1).

Lipoxygenase (LOX) Inhibition Activity

The lipoxygenase (EC 1.13.11.12) enzyme is involved in the catalysis of metabolic reactions of xenobiotics and is also associated with the conversion of polyunsaturated fatty acids and lipids to leukotrienes which results in inflammation. Lipoxygenase has also a significant role in the growth of cancerous cells, invasiveness, metastasis, tumor necrosis factor (TNF) induction and survival of cells. The compounds **8-13** were screened and compound **10** was found as a potent lipoxygenase inhibitor but compound **11** reflected activity to a significant level. In this regard, compounds **9**, **12-13** exhibited moderate activity in comparison with the standard but **8** has no significant activity (Table 1).

α-Glucosidase Inhibition Activity

The terminal non-reducing 1-4 linkage of α- glucose residues is hydrolyzed by α-glucosidase (EC 3.2.1.20) for releasing α- glucose molecule from the glucosides. The glucosidic linkage is hydrolyzed by catalysis through every carbohydrate-hydrolase results in the products which may have either retained or inverted configuration at the anomeric carbon. The subsite affinities of active site of enzyme make α-glucosidase a substrate selective enzyme. The nucleophilic displacement mechanism and a mechanism involving oxocarbenium ion intermediate were significant models which were presented for the catalytic mechanisms of carbohydrate -hydrolases. The potent *α-glucosidase* inhibitors among synthesized compounds were **8-10** and **13** while significant activity was found for compound **12**. However, compound **11** showed a non significant activity (Table 1).

Antibacterial Activity

Bacillus subtilis is one of the members of Gram-positive organism and its commonly found habitat is soil and vegetation and ropiness is associated with this organism. *Staphylococcus aureus* is a pathogen found in wound infections, bone joint, skin, endovascular and soft tissues and is gram-positive, facultative anaerobic coagulase-positive catalase-positive potential pathogen. The minor skin infections like scalded skin syndrome and cellulitis folliculitis to life-threatening diseases like pneumonia, sepsis and toxic shock syndrome are caused by *Staphylococcus aureus*. Methicillin-resistant *S. aureus* is one of greatly feared strains of *S. aureus*, which have become resistant to most antibiotics. *Escherichia coli* is non-sporulating, facultative anaerobic, gram negative bacterium, and is mostly present in lower intestine of warm-blooded organisms. Some bacterial infections including cholecystitis, bacteremia, cholangitis, pneumonia, urinary tract infection (UTI), vomiting and bloody diarrhea and food poisoning is associated with some serotypes but most of the strains are harmless.

Salmonella typhi is facultative anaerobic pathogen, gram negative bacteria and causes the typhoid or enteric fever, which is the cause of sixty thousands of deaths in the world and affects 17 million people on yearly basis. *Pseudomonas aeruginosa* is rod-shaped, non-sporulating, facultative anaerobic pathogen and infection in urinary tract, lungs, and kidneys are associated with this pathogen.

The Schiff bases **8-13** were screened against *B. subtilis, S. aureus, E. coli, S. typhi* and *P. aeruginosa* bacteria by employing agar well diffusion method. All the compounds reflected good activity against all bacteria except *P. aeruginosa* (Table 2).

CONCLUSION

The Schiff bases **8-13**, have been synthesized by the condensation of 2-aminophenol **1** with aldehydes/kitones **2-7** and were biological screened out and showed good lipoxygenase, α-glucosidase inhibition and antioxidant activities, whereas compound **10** showed potent lipoxygenase inhibition activity.

The compounds **8-10** and **13** were found as potent α-glucosidase inhibitors. The compounds **9** and **13** were found as potent antioxidant. All the compounds were found to have good activity against bacterial strains but had no significant activity against urease enzyme.

Table 1: IC_{50} (µM) values for Schiff bases **(8-13)** in the antioxidant, urease, lipoxygenase and α-glucosidase assays.

Compound	DPPH Scavenging Activity IC_{50} (µM)	Urease Inhibition Activity IC_{50} (µM)	Lipoxygenase Inhibition Activity IC_{50} (µM)	α-Glucosidase Inhibition Activity IC_{50} (µM)
8	107	> 115	> 115	11.8
9	22.7	> 115	111.8	15.1
10	68.3	> 115	12.3	10.7
11	110.2	> 115	78.6	> 115
12	114.7	> 115	113.4	46.1
13	28.9	> 115	107.1	36.4
BHA	44.2	-	-	-
Baicalein	-	-	22.6	-
Thiourea	-	21.6	-	-
Acarbose				39.0

(> 115, Non-significant)

Table 2: The values of diameter of the zone inhibition for Schiff bases **(8-13)** in the Antibacterial Assay.

Bacteria	Gentamicin (0.3%)	8	9	10	11	12	13
	Zone inhibition (mm)	Zone inhibition (mm)	Zone inhibition (mm)	Zone inhibition (mm)	Zone inhibition (mm)	Zone inhibition (mm)	Zone inhibition (mm)
B. subtilis	28	23	24	25	17	21	23
S. aureus	30	29	21	27	27	11	27
E. coli	25	15	14	17	19	7	18
S. typhi	28	5	13	22	17	13	21
P. aeruginosa	30	7	4	10	2	11	5

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