Study of Antioxidant, Anti-protease and Anti-urease Potential of Schiff Bases of Acetophenone with Different Amines

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Summary: Seven acetophenone-derived Schiff bases were synthesized with different amines including 2-aminobenzoic acid (HL1), 4-aminobenzoic acid (HL2), 2-naphthylamine (HL3), phenylhydrazine (HL4), 1,2-ethanediamine (HL5), 1,2-diaminobenzene (HL6) and 1,4-diaminobenzene (HL7), and were subjected to various assays including FRAP (ferric reducing antioxidant power), DPPH (1,1-diphenyl-2-picrylhydrazyl), phosphomolybdate, reducing power, and lipid peroxidation inhibition. They were also evaluated for protease and urease inhibitory activities. Based on the results, structure-activity relationship (SAR) was determined. Only two bases, HL1 and HL4, exhibited antioxidant or free radical scavenging activity in DPPH assay. HL4 was most potent (IC_{50} 15 µg/mL), while HL1 was only slightly active. As HL4 was the only base with hydrogen bonded to nitrogen, most probably it involves hydrogen transfer (HT) mechanism. All the bases exhibited a range of antioxidant activity in assays involving electron transfer (ET). In the reducing power assay, HL5, HL6 and HL7 showed considerable potential while in FRAP assay, HL7 was most active followed by HL3. In phosphomolybdate assay, HL6 had the highest potency followed by HL7, while HL4 and HL3 also displayed good activity. All the bases showed moderate to high lipid peroxidation inhibitory activity. HL7 exhibited highest protease inhibitory activity (EC_{50} 43.9 µg/mL) followed by HL6 (EC_{50} 52 µg/mL). HL4 and HL5 did not show protease inhibitory activity at all. HL2 was most potent in inhibiting urease activity (EC_{50} 29.91 µg/mL). HL5 and HL6 showed moderate activity. The study showed how variation in structures of Schiff bases alters their antioxidant and anti-enzymatic activities.

Keywords: Schiff bases; Acetophenone; Anti-enzymatic; Antioxidant; Proteases; Ureases.

Introduction

Reactive oxygen species (ROS), which are often produced as side products of chemical reactions in human body, exert damaging impact on living cells [1, 2]. A condition called oxidative stress is caused when the natural antioxidant defence system fails to scavenge free radical produced in the body [3, 4]. The oxidative stress can cause disorders of acute severity [5-8] like cancer, cardiovascular diseases, hypertension, atherogenesis, Alzheimer's and Parkinson's diseases. Since scavenging of free radicals involves different mechanisms, a variety of assays have been developed to study the antioxidant efficacy of new drug candidates. These include FRAP (ferric reducing antioxidant power), phosphomolybdate, DPPH (1,1-diphenyl-2-picrylhydrazyl), reducing power, and lipid peroxidation inhibition assays. Over the past few decades, numerous Schiff bases have been synthesized and evaluated for different bioactivities [9, 10]. Many Schiff bases have been reported as excellent antioxidant agents [11, 12].

Urease is reported to have a role in the growth of urolithiasis, ammonia and hepatic encephalopathy, hepatic coma, pyelonephritis and urinary catheter encrustation [13, 14]. It causes infections generated by bacterium *Helicobacter pylori*, making survival of the microorganism possible at low pH of the stomach during colonization. Thus, it amplifies the risk of gastric and peptic ulcer leading to cancer [15, 16]. In agriculture, urease activity, during urea fertilization, can pose severe environmental and economic issues by excessive release of ammonia into the atmosphere. This deprives plant of essential nutrients and increases the pH of soil causing ammonia toxicity [17, 18]. Hence, urease inhibition may be used as a strategy for the treatment of gastro-intestinal ulcers caused by *H. pylori*, and a mechanism to ensure optimum availability of urea fertilizer for vegetation.

Proteases, the largest group of enzymes, are accountable for physical, chemical and enzymatic modification of proteins [19]. Protease inhibition of pathogenic organisms may aid in control of several degenerative diseases [20, 21]. Schiff bases and their metal complexes have a promising history in protease inhibition [22].

In the present work, antioxidant potential, and protease and urease inhibitory activities of seven acetophenone-derived Schiff bases are reported. The

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Schiff bases HL1-HL7 (Fig. 1) were synthesized by reacting acetophenone with 2-aminobenzoic acid, 4-aminobenzoic acid, 2-naphthylamine, phenylhydrazine, 1,2-ethanediamine, 1,2-diaminobenzene, and 1,4-diaminobenzene, respectively, and characterized as reported earlier [23].

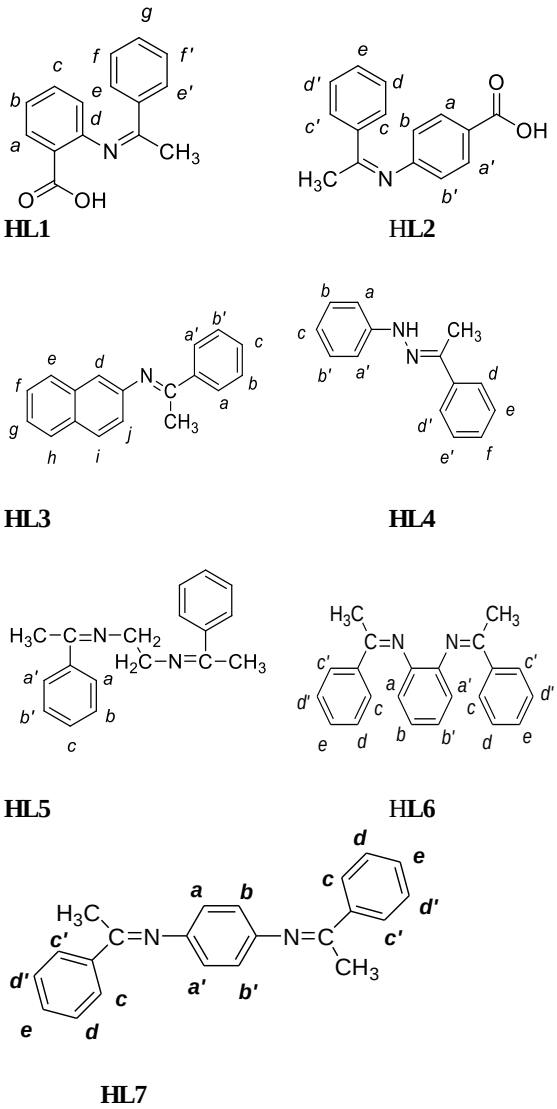

Fig. 1: Structures of Schiff bases [23] evaluated for antioxidant and enzyme inhibitory activities.

Results and Discussion

DPPH Radical Scavenging Activity

DPPH (2,2-diphenyl-1-picrylhydrazyl) is a stable free radical that has a UV-Vis absorption maximum at 517 nm, and whose purple colour fades upon reduction (to yellow) when allowed to react with an antioxidant [24]. The concentration of an antioxidant that causes a 50% decrease in DPPH concentration is defined as IC_{50}. The DPPH radical is believed to react by either electron transfer (ET) or hydrogen transfer (HT) mechanism. Our results (Table-1) clearly supported the HT mechanism, as only those two Schiff bases responded to the assay in which transfer of H radical was possible. The HL4, which has hydrogen bonded to nitrogen, showed good radical scavenging potential with IC_{50} of 15 µg/mL. HL1, however, showed a very high IC_{50} value (520 µg/mL) compared with that of ascorbic acid under the similar conditions (4.91 (µg/mL). Interestingly, HL2 which is a constitutional isomer of HL1 was inactive against DPPH radical. It may be hypothesized that in HL1, the carboxylic hydrogen is bonded to imine nitrogen through intra-molecular hydrogen bonding. It is therefore less acidic and can be removed as free radical.

The rest of the compounds had no free hydrogen to be lost for neutralization of DPPH radical. Hence, they remained highly inactive in the assay.

Table-1: Antioxidant activities of acetophenone-derived Schiff bases determined by different assays.

Bases	DPPH IC_{50} (µg/mL)	FRAP Assay µg/mL of AAE*	RP Assay^	$^{+}$PM Assay µg/Ml of AAE*
HL-1	520	37.57	0.070	46.91
HL-2	---	29.50	0.060	47.33
HL-3	---	61.80	0.156	77.33
HL-4	15	39.88	0.069	92.75
HL-5	---	24.88	3.082	46.50
HL-6	---	32.96	3.363	377.33
HL-7	---	83.34	3.400	109.83

*AAE ascorbic acid equivalents; $^{+}$phosphomolybdate; ^reducing power activity of solutions having concentration 1 mg/mL, activity of BHT was 1.200; IC_{50} of ascorbic acid was 4.91

FRAP Assay

The FRAP assay is based on reduction of ferric ions (Fe^{3+}) to ferrous ions (Fe^{2+}) by an antioxidant to form a coloured ferrous-tripyridyltriazine complex [25]. The FRAP assay involves the electron transfer mechanism. Ascorbic acid was used as standard and FRAP values were calculated using the equation derived from its standard curve.

Absorbance = 0.0026(Concentration of ascorbic acid equivalent) + 0.0123

Concentration of ascorbic acid equivalent = (Absorbance – 0.0123)/ 0.0026

In this assay, the FRAP values of the Schiff bases HL1, HL2, HL3, HL4, HL5, HL6, and HL7 were, respectively, 37.57, 29.50, 61.80, 39.88, 24.88, 32.96, and 83.34 µg/mL of AAE (Table-1). The higher values of HL7 and HL3 may be attributed to greater resonance stability of their free radicals.

Reducing Power Assay

In the reducing power assay, the sample antioxidants reduce Fe^{3+} to Fe^{2+} by donation of an electron resulting in the formation of a blue coloured iron(II) complex [26]. The activity was highest for HL7 and lowest for HL2 (Table-1). High absorbance indicated high reducing power due to the strong antioxidant potential. In our studies, HL5, 6, and 7 had higher reducing power than that of BHT, the standard antioxidant used.

Antioxidant activity by Phosphomolybdate

The phosphomolybdate antioxidant assay follows the reduction of molybdenum(VI) to molybdenum(V) by the antioxidant sample and is detected by the formation of green coloured molybdenum(V) complex at an acidic pH [27]. In our tests, the antioxidant activity of the compounds ranged from 46.5-377.3 µg/mL of AAE (Table-1). The HL6 showed the highest while the HL5 showed the lowest antioxidant potential.

Lipid Peroxidation Inhibitory Assay

Highly reactive peroxides are formed upon peroxidation of linoleic acid having the potential to oxidize Fe^{2+} to Fe^{3+}. When thiocyanate ions react with Fe^{3+}, a complex is formed which is detected at 500 nm [28].

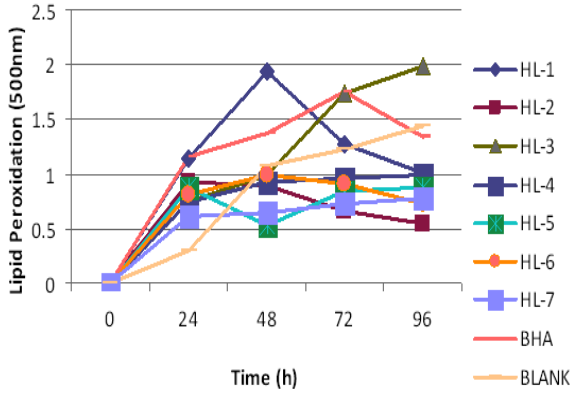

Fig. 2: Lipid peroxidation inhibitory activity of acetophenone derived Schiff bases in comparison with standard BHA.

Linoleic acid oxidation is retarded by the action of oxidants thus decreasing the production of peroxides which in turn leads to low lipid peroxidation value indicating high antioxidant potential. Lipid peroxidation as a function of time is plotted in Fig. 2. BHA was used as standard antioxidant

Urease Inhibitory Activity

Percent urease inhibitory activities of the acetophenone-derived Schiff Bases were measured using a reported protocol [29], and the results are presented in Table-2. The bases demonstrated a varying degree of urease inhibitory properties at different concentrations. As the table shows, HL3 remained highly inactive against urease within a broad concentration range. At the concentration of 50 µg/mL, HL1, HL2, HL3, HL4, HL5, HL6, and HL7 showed 27, 70, 0, 36, 53, 54, and 36% urease inhibitory activity, respectively. The EC_{50} values of HL1, HL2, HL4, HL5, HL6, and HL7 were 90.01, 29.91, 70.17, 49.95, 50.00, and 80.02, respectively. Notably, the activity of HL2 was almost comparable to that of thiourea (9.66 µg/mL).

Protease Inhibitory Activity

The protease inhibitory activities of the acetophenone derived Schiff bases were measured according to a reported protocol [30]. The bases exhibited a varying degree of protease inhibitory properties at different concentrations. As the Table-2 shows, HL4 and HL5 remained highly inactive against protease within a broad range of concentration. At the concentration 50 µg/mL,HL1, HL2, HL3, HL4, HL5, HL6, and HL7 showed 16.8, 30.7, 33.0, 0, 0, 48.4, and 61.4 percent activity, respectively. The EC_{50} values of HL1, HL2, HL3, HL6, and HL7 were 105, 90.2, 65.9, 52.4, and 43.9, respectively. Thus, HL7 was most effective and its value was somewhat comparable to that of the standard Ritonavir (36.5 µg/mL). Therefore, it may be considered as a moderate anti-protease agent.

Table-2: Percent protease and urease inhibitory activities and EC_{50} values of acetophenone-derived Schiff bases.

Schiff Base	Protease		Urease	
	% Inhibitory Activity	EC_{50} (µg/mL)	% Inhibitory Activity	EC_{50} (µg/mL)
HL-1	**16.8**	**105.0**	**27.0**	**90.01**
HL-2	**30.7**	**90.2**	**70.0**	**29.91**
HL-3	**33.0**	**65.9**	**0.0**	**----**
HL-4	**0.0**	**----**	**36.0**	**70.17**
HL-5	**0.0**	**----**	**53.0**	**49.95**
HL-6	**48.4**	**52.4**	**54.0**	**50.00**
HL-7	**61.4**	**43.9**	**36.0**	**80.02**
Standard	---	**36.5**	---	**9.66**

Standard for protease inhibitory activity was Ritonavir and for urease inhibitory activity thiourea

Experimental

Materials and Methods

Chemicals

Ascorbic acid and DPPH (1,1-diphenyl-2-picrylhydrazyl) radical were purchased from MP Biomedicals (France). Folin-Ciocalteu reagent, ammonium molybdate, iron(II) sulfate, iron(II) chloride and butylated hydroxyanisole (BHA) were purchased from Merck (Germany) and gallic acid from Scharlau (Switzerland). Linoleic acid and TPTZ (2,4,6-Tripyridyl-s-triazine) were obtained from Sigma-Aldrich (Germany). Potassium ferricyanide and trichloroacetic acid were of Unichem (China). Urease (Jack Bean) was purchased from Avonchem. All the reagents and solvents employed in this work were of analytical grade. To record absorbance, UV-visible spectrophotometer UVD-3200 Labomed, Inc. was used.

Synthesis and Characterization of Schiff Bases

The Schiff bases of acetophenone employed in this study were synthesized and characterized as reported earlier [23]. They were synthesized using the conventional reflux method, and their structures were determined on the bases of ^{1}H and ^{13}C NMR, infrared (IR) and mass spectroscopic data.

DPPH Radical Scavenging Assay

The DPPH free radical scavenging assay was conducted as per the protocol reported by Brand-Williams *et al*. [24]. To prepare stock solution of the DPPH radical, 24 mg DPPH was dissolved in 100 mL methanol. The solution was kept in a refrigerator, covered with an aluminium foil until used. DPPH working solution was prepared by diluting 20 mL of the DPPH stock solution with methanol to obtain the absorbance of about 0.97 (±0.03) at 517 nm. Different dilutions of synthesized Schiff bases were prepared in methanol. Ascorbic acid was used as a standard antioxidant. In a test tube, the DPPH working solution (3 mL) was mixed with a Schiff base solution (100 µL) and the mixture was incubated at 37 °C for 30 min. Absorbance of the mixture was recorded against blank (methanol) at 517 nm. The antioxidant activity of each sample was calculated as per the equation given below:

$$\%\text{Antioxidant Activity} = 100[(1 - (As/Ac)]$$

where, As (sample absorbance) is the absorbance by 3 mL DPPH working solution + 100 µL Schiff base, while Ac (control absorbance) is the absorbance by 3 mL DPPH working solution + 100 µL methanol. Based on change in free radical scavenging activity of a sample with concentration, IC_{50} values were calculated.

FRAP (Ferric Reducing Antioxidant Potential) Assay

The total antioxidant activity of Schiff bases was determined by FRAP assay of Benzie and Strain [25]. To prepare 10 mM TPTZ solution, 0.031 g of TPTZ was dissolved in hydrochloric acid (10 mL, 40 mM). It was heated on a water bath at 50 °C for 5 min. To prepare 20 mM ferric chloride solution, 0.05406 g $FeCl_3.6H_2O$ was dissolved in distilled water (10 mL). Fresh FRAP reagent was prepared by mixing acetate buffer (25 mL, pH 3.6), TPTZ solution (2.5 mL), and ferric chloride solution (2.5 mL). Then the mixture was incubated at 37 °C for 15 min before use. Different dilutions of each Schiff base were prepared in methanol. Aqueous solution of ascorbic acid was used as a standard. In a cuvette, 2.9 mL FRAP reagent and 100 µL Schiff base solution were mixed and incubated for 30 min in the dark. The absorbance of the mixture was measured against the blank (2.9 mL FRAP reagent in 100 µL methanol) at 593 nm. The FRAP value was expressed as µg/mL of ascorbic acid equivalents.

Reducing Power Assay

The reducing power assay was carried out by the protocol of Oyaizu [26]. Solution of compound or Gallic acid (2.5 mL) was mixed with 0.2 M sodium phosphate buffer (2.5) and 1% potassium ferricyanide (2.5 mL). The mixture so obtained was incubated at 50 °C for 20 min, followed by addition of trichloroacetic acid solution (2.5 mL, 100 mg/L). It was centrifuged at 650 rpm for 10 min, and the supernatant (5 mL) was diluted with distilled water (5 mL). Then, 1 mL ferric chloride (0.1%) solution. The absorbance of the mixture was determined at 700 nm.

Phosphomolybdate Antioxidant Assay

The phosphomolybdate antioxidant assay was carried out using the procedure reported by Umamaheswari and Chatterjee [27]. Phosphomolybdate reagent was prepared by mixing measured amounts of ammonium molybdate (100 mL, 4 mM), sulfuric acid (100 mL, 0.6 M), and sodium phosphate (100 mL, 28 mM) solution. In a test tube, phosphomolybdate reagent (3 mL) was mixed with 300 µL solution of the compound or standard (ascorbic acid) or methanol. The test tubes, capped with silver foil, were incubated on a water bath at 95 °C for 90 min. The test tubes were allowed to come to room temperature before recording the absorbance at 765 nm. The antioxidant activity was expressed as µg/mL of ascorbic acid equivalents.

Lipid Peroxidation Assay

The lipid peroxidation inhibitory activities of the Schiff bases were determined by following the assay reported by Mitsuda [28]. Linoleic acid emulsion was prepared by mixing Tween 20 (175 µg) to linoleic acid (155 µL), and adding potassium phosphate buffer (pH 7, 0.05 M) to obtain 50 mL volume. Then, a sample solution (100 µL) was mixed with potassium phosphate buffer (2.4 mL) and linoleic acid emulsion (2.5 mL). The mixture so obtained was incubated for 25 min at 37 °C. This mixture was analysed for four days by taking 100 µL at 24 h intervals. The mixture was dissolved in ethanol (3.7 mL), and treated with iron(II) chloride solution (100 µL, 20 mM). Finally, 100 µL potassium thiocyanate solution (30%) was added and mixed. Absorbance of the mixture was noted at 500 nm on a spectrophotometer. Blank consisted of linoleic acid emulsion (2.5 mL) and potassium phosphate buffer (2.5 mL). BHA (butylated hydroxyanisole) was used as a standard.

Anti-Urease Assay

The phenol hypochlorite anti-urease assay reported by Ghous *et al*. was adopted [29]. In the method, the enzyme urease is used to catalyze the conversion of urea to ammonia. The ammonia so released is allowed to react with salicylate, hypochlorite and nitroprusside. A blue-green dye (indophenols) is produced which is monitored at 625 nm. The absorbance is proportional to the concentration of NH_3 produced in the reaction, which, in turn, is the measure of enzymatic activity. The reagent R1 is made by mixing 5 different solutions in equal volume. They are 0.12 M phosphate buffer (pH 7, prepared by dissolving 1.62 g potassium dihydrogenphosphate in 100 mL distilled water and adjusting its pH by the addition of 25% potassium hydroxide solution), 0.06 M aqueous sodium salicylate, 0.005 M aqueous sodium nitroprusside (0.148 sodium nitroprusside dissolved in 100 mL distilled water), 0.001 M aqueous EDTA (0.037 g EDTA dissolved in 100 mL distilled water) and the enzyme (0.0005 g urease in 100 mL distilled water). The reagent R2 consisted of equal volumes of 0.12 M phosphate buffer, 0.4 M sodium hydroxide solution and 0.01 M sodium hypochlorite solution.

Following formula was used to calculate % inhibition:

% Inhibition = 100(Control Absorbance - Test Absorbance)/Control Absorbance

Different dilutions of the given Schiff base and thiourea were prepared in 0.12 M phosphate buffer. To a dilution of the Schiff base in phosphate buffer (total volume 5 mL), 15 µL urea solution, 0.485 mL phosphate buffer and 2.5 mL R1 were mixed in a test tube and incubated at 37 °C for 5 min. Then, 2.5 mL R2 was mixed and the mixture was allowed to incubate for further 10 min at 25 °C. The absorbance was measured at 625 nm. For blank, 5.5 mL buffer, 2.5 mL R1 and 2.5 mL R2 was added. For control, 15 µL urea solution, 5.485 mL buffer and 2.5 mL R1was added and incubated at 37 °C for 5 min. Then, 2.5 mL R2 was mixed and incubated for further 10 min at 25 °C. The protocol is given in Table-3.

Anti-Protease Assay

Anti-protease assay reported by Sigma [30] was adopted. Casein was used as a substrate. Upon action with protease, casein undergoes hydrolysis to produce tyrosine along with other amino acids. The released tyrosine is allowed to react with the Folin-Ciocalteu reagent which results in the formation of a coloured product, which is monitored spectrophotometrically by recording the absorbance at 660 nm. Casein solution (0.65% w/v) was prepared in potassium phosphate buffer (pH 7.5, 50 mM). The enzyme diluent consisted of sodium acetate-calcium acetate buffer (pH 7.5) and was prepared by mixing sodium acetate (10 mM) and calcium acetate (5 mM) solutions, adjusting the pH with 0.1 M acetic acid or sodium hydroxide. The protease solution (10 mg/mL) was prepared immediately before use in the enzyme diluent. Solutions of different dilutions of a given Schiff base were prepared in methanol. A drug called Ritonavir (in methanol) was used as a standard. To 5 mL pre-incubated casein (37 °C), a solution of the given Schiff base (5 mL) was mixed. The solution so obtained was incubated at 37 °C for 15 min. Enzyme solution (1 mL) was added to it, and the mixture was allowed to incubate for another 15 min at 37 °C. TCA (5 mL, 110 mM) was added to stop the enzymatic reaction. The mixture was allowed to stand for 15 min at 37 °C and filtered. To the filtrate (2 mL), sodium carbonate solution (5 mL, 500 mM) and Folin-Ciocalteu reagent (1 mL, 2 M) were added. After final incubation for 30 min at 37°C, absorbance was noted at 660 nm against a blank, which contained the same reagents except that it was added 1 mL enzyme diluent instead of the enzyme solution. The control contained 5 mL methanol in place of the inhibitor (Schiff base). The protocol is displayed in Table-4.

Table-3: Protocol of urease inhibitory study of Schiff bases prepared from acetophenone.

	Sample in buffer (mL)	Urea (mL)	Buffer (mL)	Reagent 1 (mL)	Incubation at 37°C (min)	Reagent 2 (mL)	Incubation at 25°C (min)
Test	5	0.015	0.485	2.5	5	2.5	10
Blank	---	-	5.500	2.5	-	2.5	-
Control	---	0.015	5.485	2.5	5	2.5	10

Table-4: Protocol for protease inhibitory activity assay.

Reagents (mL)	Test	Blank	Control
Casein	5.00	5.00	5.00
Incubation at 37 °C for 5 min			
Inhibitor	5.00	5.00	- - -
MeOH	- - -	- - -	5.00
Incubation at 37 °C for 15 min			
Enzyme Solution	1.00	- - -	1.00
Enzyme Diluent	- - -	1.00	- - -
Incubation at 37 °C for 15 min			
TCA	5.00	5.00	5.00
Incubation at 37 °C for 30 min followed by filtration			
Filtrate	2.00	2.00	2.00
Na_2CO_3	5.00	5.00	5.00
F-C reagent	1.00	1.00	1.00
Incubate at 37 °C for 30 min			

The percent inhibitory activity was calculated with the following formula:

% Inhibition = 100(Control Absorbance - Test Absorbance)/Control Absorbance

Conclusion

The Schiff base (*E*)-1-phenyl-2-(1-phenylethylidene)hydrazine(HL4) and N^1,N^4-bis(1-phenylethylidene)benzene-1,4-diamine (HL7) exhibited moderate free radical scavenging activity. The study showed DPPH involving probably hydrogen transfer mechanism in its radical scavenging action. The base *(Z)*-4-((1-phenylethylidene)amino)benzoic acid (HL2) possessed moderate urease inhibition activity, and therefore can be a candidate for peptic ulcer drug discovery. It can also find application in agriculture to slow down urea hydrolysis. The HL7 can be a lead compound for conditions related to protease action. The variation in structures of the Schiff bases was found to influence their bioactivities.

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