Research Article

Synthesis, Antioxidant and Antihemolytic Activities of (4-Nitro-benzylidene)-pyridin-3-ylmethyl amine

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Abstract

In the present work, (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**) was synthesized from the reaction of 3-(amino methyl) pyridine (**1**) with 4-nitrobenzaldehyde (**2**). The synthesized compound was characterized by UV-visible, FT-IR and ^{1}H-NMR spectral studies. Antioxidant activity was studied by DPPH radical scavenging, total antioxidant assay, hydroxyl radical scavenging method, nitric oxide radical scavenging method and ferrous reducing method. The new compound was screened for their antihemolytic activity. The compound scavenged DPPH radicals and hydroxyl radicals. It is also exhibited considerable amount of anti-hemolytic activity with 58% inhibition compared to standard quericitin with 74% inhibition.

Keywords: Antioxidant activity, Antihemolytic activity, Pyridine, Schiff base

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Introduction

Nowadays, there is a great interest in Schiff bases with active antioxidant property, whether they are chemically synthesized or obtained from the nature. Azomethines are intermediates for the preparation of thiazolidinones, oxadiazolines and many other derivatives [1]. Azomethines exhibit a wide range of biological activities like antimicrobial [2], ulcerogenicity and analgesic activity [3], anti-inflammatory [4], anticancer [5] etc. A large number of substituted pyridine derivatives are prepared and tested for variety of biological activities like anti HIV [6], anti-inflammatory [7], antimicrobial [8], fungicidal [9] and anticancer [10]. Antioxidant activity is related to the compound capability of protecting biological systems from the potentially harmful effect of processes involving reactive oxygen species that can cause excessive oxidation. Free radicals play a role in the pathogenesis of chronic degenerative diseases including cancer, autoimmune, inflammatory and cardiovascular and neurodegenerative diseases and aging [11-14]. It is also known that oxidative stress can be induced by a wide range of environmental factors including UV stress, pathogen invasion, herbicide action and oxygen shortage [15]. Owing to these facts, synthetic and natural compounds with potential antioxidant activity are receiving increased attention in biological research, medicine and pharmacy [16]. In this respect, the present paper reports the synthesis, antioxidant and antihemolytic activities of (4-nitro-benzylidene)-pyridin-3-ylmethyl amine.

Materials and Methods

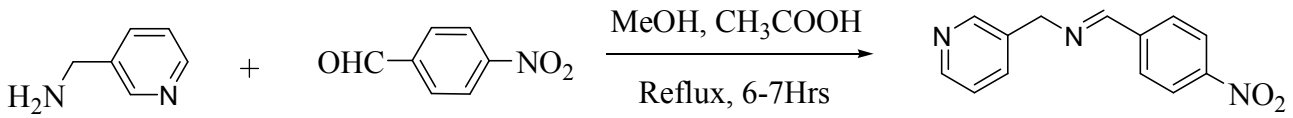
All solvents and reagents were purchased from Sigma Aldrich Chemicals Pvt Ltd and Merck. Melting range was determined by Veego Melting Point VMP III apparatus. The UV-visible spectra were recorded on Analytikjena Specord 50 UV–vis spectrophotometer with quartz cell of 1.0 cm path length in methanol. The FT-IR spectra was recorded using KBr discs on FT-IR Jasco 4100 infrared spectrophotometer and were quoted in cm^{-1}. ^{1}H NMR spectra was recorded on Bruker DMX 300, 400MHz spectrometer using DMSO-d_6 as solvent and TMS as an internal standard. Silica gel column chromatography was performed using Merck 7734 silica gel (60–120 mesh) and Merck-made TLC plates.

Experimental

Chemistry

Synthesis of (4-nitro-benzylidene)-pyridin-3-ylmethyl-amine (3)

Equimolar concentrations of 3-(aminomethyl)pyridine (**1**) (0.50 g, 0.001 mol) and 4-nitro benzaldehyde (**2**) (0.70 g, 0.001 mol) were refluxed for 6-7 hr using methanol (20 ml) and 2-3 drops of glacial acetic acid was added to the mixture. The progress of the reaction was followed by TLC until the reaction was complete. It was cooled to 0 °C, the precipitate was filtered, washed with diethyl ether and the residue was recrystallized from ethanol. FT-IR (KBr, cm^{-1}) ν: 3094 (Ar C-H), 1615 (C=N), 1502 (C=C), 1148 (C-N). ^{1}H NMR (DMSO-d_6) δ ppm: 4.31 (s, 2H, CH_2), 7.32 (t, 1H, Py-H), 7.76 (d, 1H, Py-H), 7.93 (d, 2H, Ar-H), 8.09 (s, 1H, CH=N), 8.29 (d, 2H, Ar-H), 8.62 (d, 2H, Py-H).



1 **2** **3**

Scheme 1

Biology

Antioxidant Activity

Total antioxidant capacity: Total antioxidant capacity (TCA) of the compound/standard was determined by the method reported by [17] with some modification. 0.5 ml of compound/standard at different concentrations was mixed with 3 ml of reaction mixture containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 1% ammonium molybdate into the test tubes. The test tubes were incubated at 95 °C for 10 minutes to complete the reaction. The absorbance was measured at 695 nm using a spectrophotometer against blank after cooling at room temperature. Ascorbic acid was used as standard. A typical blank solution contained 3 ml of reaction mixture and the appropriate volume of the same solvent used for the compound/standard was incubated at 95 °C for 10 minutes and the absorbance was measured at 695 nm. Increased absorbance of the reaction mixture indicated increase total antioxidant capacity.

DPPH free radical-scavenging activity: The compound was tested with DPPH free radical according to the method previously defined by Habila *et al*., [18] with some modifications. Shortly, 0.5 ml of synthesized compound and synthetic antioxidant compound (BHA) prepared at different concentrations were taken into test tubes and stirred with 2.5 ml of 2 mM DPPH solution. The mixture was stirred thoroughly and incubated for 30 min in dark laboratory conditions. The absorbance at a wavelength of 517 nm was measured by UV spectrophotometry. To determine DPPH free radical scavenging activity (FRSA) was used in the following equation.

$$FRSA\ (\%) = [(A_0\text{-}A_1)/A_0]\ x100$$

A_0 is the absorbance values without compound and A_1 the absorbance in the presence of compound. As opposed to increasing concentration of compound decline of absorbance is an indication that destroyed DPPH radical. Antioxidant activity results are expressed as IC_{50} value that reduces by half the effective concentration of DPPH radicals and was calculated by interpolation from linear regression analysis.

Scavenging capacity of hydroxyl radical: The hydroxyl radical-scavenging ability of compounds were determined using 2-deoxy-D-ribose oxidative degradation mediated by hydroxyl radicals as described by Halliwell *et.al*., [19]. The reaction mixture contained various dilutions of the compounds in DMSO (final concentration 0-100 μM; 0.5 mL), 0.1 ml of 2.8 mM 2-deoxy-D-ribose, 0.2 ml of a premixed 100 μM $FeCl_3$ and 104 μM EDTA solution (1:1, v/v), 0.1 ml of 1 mM hydrogen peroxide and 0.1 ml of 0.1 mM ascorbic acid, was incubated at 37 ˚C for 60 min. Under the present experimental conditions, DMSO did not interfere with the assay. Thereafter, 1.0 ml of 0.5% (w/v)

thiobarbituric acid in 10% (w/v) trichloroacetic acid was added and the mixtures were vortexed and heated in a water bath at 100 ˚C for 15 min. The reaction was stopped by a 5 min ice water bath. The mixtures were centrifuged at 12,000xg for 5 min at room temperature and the absorbance of the supernatant was measured at 532 nm. The scavenging effect of hydroxyl radicals (%) was calculated using the following:

$$\text{Scavenging effect (\%)} = (1\text{-Acompound/Acontrol}) \times 100$$

where Acompound is the absorbance in the presence of the tested compound, and Acontrol is the absorbance of the control contained all the reaction reagents except the tested compound. Ascorbic acid was used as the reference compound.

Nitric oxide radical scavenging assay: Nitric oxide generated from sodium nitroprusside in aqueous solution at physiological pH 7.4 spontaneously generates nitric oxide which interacts with oxygen to produce nitric ions that can be estimated by using Griess reagent. Scavenging nitric oxide compete with oxygen leading to reduce production of nitric ions. 1ml of sodium nitroprusside (10 mM) in phosphate buffer saline (0.2M, pH 7.4) was mixed with 100 ml compound solution of various concentrations (0, 20, 40, 60, 80, 100 µg) and incubated at room temperature for 150 mins. The same reaction mixture without the compound was used as the control, after the incubation period 0.5 ml of griess reagent (1% sulfanilamide, 2% H_3PO_4 and 0.1% N-(1-napthyl)ethylenediaminedihydrochloride) was added. The absorbance of the chromophore (pink colour) formed was read at 546 nm. The scavenging activity was calculated using the formula.

$$\text{\% of radical scavenging activity} = \frac{\text{Absorbance of control} - \text{Absorbance compound}}{\text{Absorbance of control}} \times 100$$

Assay of reducing power: The reducing power was determined according to the method of Oyaizu et. al., [20] with slight modifications various concentrations of triazole derivatives (2.5 ml) were mixed with 2.5 ml of 200 mM sodium phosphate buffer (pH 6.6) and 2.5 ml of 1% potassium ferricyanide. The mixture was incubated at 50 ºC for 20 min. After 2.5 ml of 10% trichloroacetic acid (w/v) were added, the mixture was centrifuged at 1000 rpm for 8 min. The upper layer (5 ml) was mixed with 5 ml of deionized water and 1 ml of 0.1% of ferric chloride, and the absorbance was measured spectrophotometrically at 700 nm. The assays were carried out in triplicate and the results expressed as mean values ± standard deviations. BHA, BHT and α-tocopherol were used as standards. It was indicated that high absorbance of the compound was good reducing power in the reaction conditions.

Antihemolytic Assay

Erythrocyte suspension was prepared [21] and human blood compound was collected in glass centrifuge tubes with known amount of anticoagulant from a well-nourished healthy person. It was centrifuged at 1000 rpm for 10 min and erythrocytes were separated from the plasma and washed three times. The erythrocytes separated were then diluted with phosphate buffer saline (0.2m, pH 7.4) to give 4% suspension. To that 2 ml of the erythrocyte suspension 50-500 mg/ml of buffer saline was added and the volume was made up to 5 ml with buffer saline. The mixture was incubated for 5 min at room temperature and then 0.5 ml of H_2O_2 solution in buffered saline was added to induce oxidative degradation of the membrane lipid (haemolysis). In another set quercitin (50-500 mg/ml) and was taken as a reference compound and treated in the similar manner. Therefore the tubes were centrifuged at 1000 rpm for 10 mins and the colour density of the supernatant was measured spectrophotometrically at 580 nm.To obtain 100% haemolysis (control) 2 ml of distilled water was added to 2 ml of RBC suspention. The relative hemolysis in the control was taken as 100%. Inhibitory activity of the compound on haemolyte was calculated and expressed as present haemolysis.

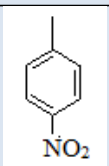
$$\text{\% Hemolysis} = \frac{\text{Absorbance of control} - \text{Absorbance of compound}}{\text{Absorbance of control}} \times 100$$

Result and Discussion

Chemistry

Schiff bases of (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**) was synthesized from the reaction of 3-(aminomethyl)pyridine with 4-nitrobenzaldehyde was confirmed by recording their ^{1}H NMR, FT-IR and UV-visible spectra. The chemical structure and physical data of synthesized compound is tabulated in **Table 1**. The UV spectra of **3** were recorded using suitable solvent in the range of 200 - 800 nm. The electronic absorption spectra of compounds show new bands and appearance of wavelength absorption band in the UV region in UV-visible spectrum owing to confirms the formation of new compounds. The FT-IR spectra of **3** were recorded using KBr pellets in the range of 4000 - 400 cm^{-1}. The absence of NH_2 and HC=O absorption bands in the IR spectra confirmed that the synthesized compounds. The absorption band at 3094 cm^{-1} has been assigned to the aromatic-H stretch. The appearance of a medium to strong absorption band at 1615 cm^{-1} due to a stretching vibration of the azomethine (HC=N) bond formation in the synthesized compound. The absorption band at 1502 cm^{-1} is due to the C=C stretch. The band appeared at 1148 cm^{-1} corresponding to C-N stretching frequency. The characteristic resonance peaks in ^{1}H NMR for the synthesized compound **3** was reported using DMSO-d_6. The proton spectral data agree with respect to the number of protons and their chemical shifts with the proposed structures. The proton spectral data of the starting material, 4-nitrobenzaldehyde (**2**) shows resonance at δ 10.01 ppm (s, 1H, CHO). In the synthesized compound **3** the above resonance disappeared and additional resonance assigned to the –CH=N (δ 8.09 ppm) was observed which confirmed the product.

Table 1 Chemical structure and physical data of the synthesized compound

Compound	R	Molecular Formula	Mol. Wt.	Solubility	M.R. (°C)	UV-visible (nm)	Yield (%)
3		$C_{14}H_{12}N_2O_2$	240.2	Ethanol	140-142	228	68.4

Biology

In living system, free radicals are constantly generated and they can cause extensive damage to tissue and biomolecules leading to various disease conditions, especially degenerative disease and extensive lysis. The percentage of antioxidant assay of the compound **3** is given below in **Tables 2** and **3.** Many synthetic drugs protect against oxidative damage, compound **3** ((4-nitro-benzylidene)-pyridin-3-ylmethyl amine) is one such compound used in this present study shows that it has the potent antioxidant property (**Figures 1-5**). It shows moderate free radical scavenging property. The lysis of erythrocytes in presence of water and hydrogen peroxide was considered as 100% hemolytic activity and the pyridine containing compound (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**). Compound shows considerable antihemolytic activity (**Figure 6**) by showing lesser lysis of RBCs in **Table 4**.

Table 2 The percentage of scavenging activity of the (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**)

Types of Antioxidant	Compound	% of scavenging activity				
		200 µg	400 µg	600 µg	800 µg	1000 µg
Total antioxidant assay	**3**	12±0.33	28±0.36	42±0.25	53±0.21	73±0.49
	Ascorbic acid	18±0.22	36±0.34	54±0.45	72±0.56	89±0.62
DPPH assay	**3**	9± 0.22	17±0.41	25±0.47	33±0.23	41±0.53
	Ascorbic acid	10±0.34	19± 0.26	28± 0.24	36± 0.40	40± 0.36
Ferrous Reducing Antioxidant Assay	**3**	21±0.33	29±0.39	33±0.51	88±0.39	89±0.41
	Ascorbic acid	15±1.42	30±1.32	43±1.43	60±1.25	75±1.54
Nitric Oxide Scavenging Activity	**3**	18±0.53	27±0.41	34±0.62	39±0.58	54±0.28
	Ascorbic acid	20± 0.32	38± 0.34	60± 0.39	80± 0.36	95± 0.41

Table 3 The percentage of hydroxyl radical scavenging activity of the (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**)

Compound	% of scavenging activity in µg
3	45 ± 0.63
Ascorbic acid	67 ± 0.52

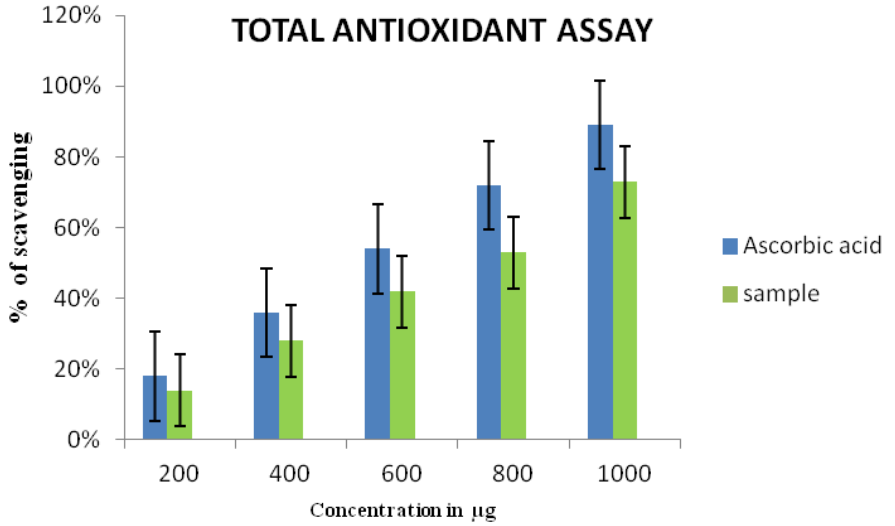


Figure 1 Total antioxidant assay

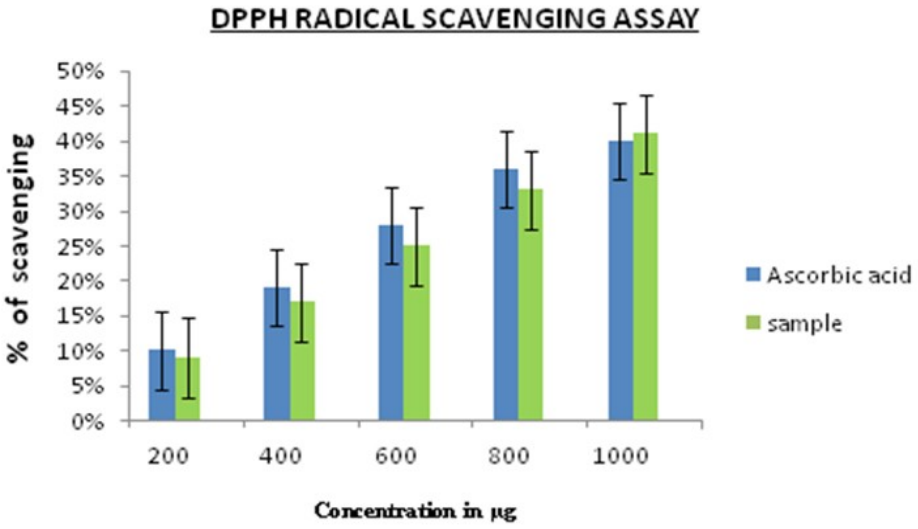


Figure 2 DPPH antioxidant assay

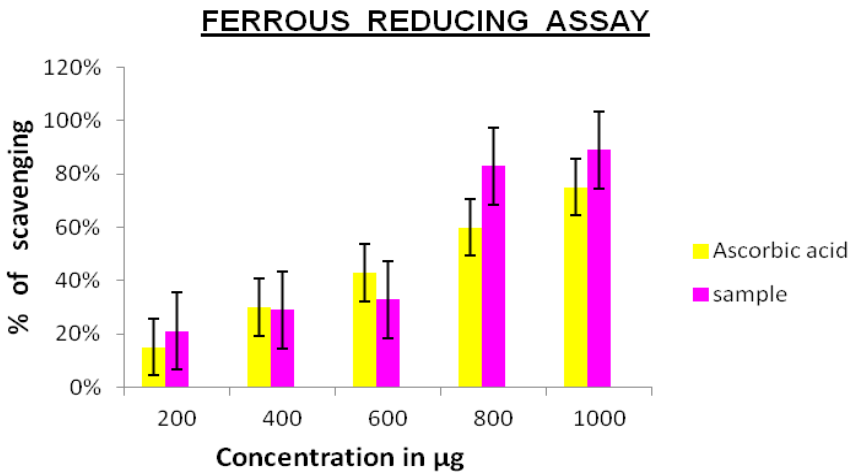


Figure 3 Ferrous reducing assay

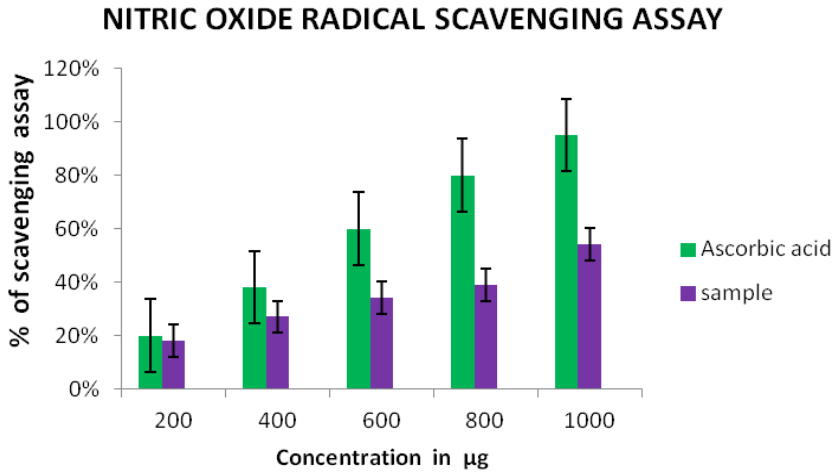


Figure 4 Nitric oxide radical scavenging assay

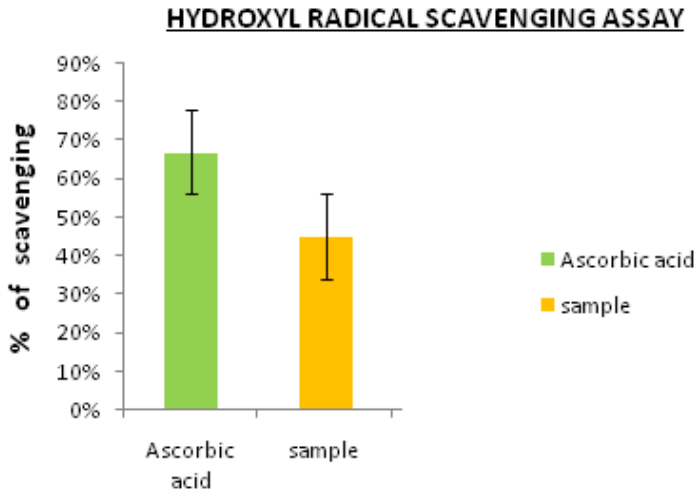


Figure 5 Hydroxyl radical scavenging assay

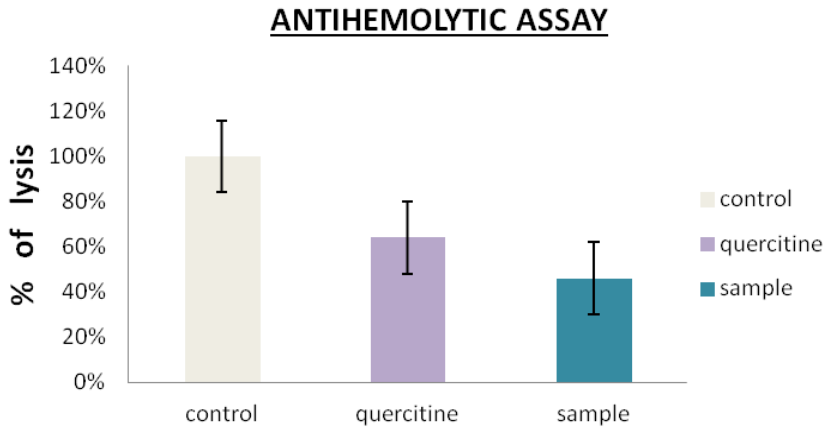


Figure 6 Anti-hemolytic activity

Table 4 The percentage of lysis of the (4-nitro-benzylidene)-pyridin-3-ylmethyl amine (**3**)

Compound	% of lysis
3	58 ± 1.2
Control	100 ± 1.8
Quercitin	74 ± 1.6

Conclusion

The synthesized (4-nitro-benzylidene)-pyridin-3-ylmethyl amine significantly exhibited antioxidant activity such as total antioxidant assay, DPPH, hydroxyl radicals scavenging, ferrous reducing and nitric oxide radicals, the compound scavenged DPPH radicals and hydroxyl radicals. The synthetic compound (4-nitro-benzylidene)-pyridin-3-ylmethyl amine also exhibited considerable amount of anti-hemolytic activity with 58% inhibition compared to standard quericitin with 74% inhibition.

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