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Research Article

Synthesis and Characterization of N^1-Phenylhydrazine-1,2-*bis*(carbothioamide) and Its Evaluation for Antimicrobial, Antioxidant, and Brine Shrimp Lethality Bioassay

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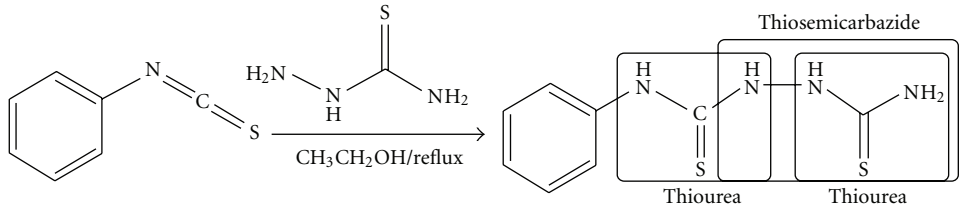
The compound N^1-phenylhydrazine-1,2-*bis*(carbothioamide) was synthesised from phenylisothiocyanate reacting with thiosemicarbazide refluxing the mixture in ethanol. The new compound obtained was characterised by various spectral and elemental analyses. It was subjected to antibacterial, antioxidant and brine shrimp lethality bioassay. The compound showed brine shrimp lethality with LC_{50} value of 12.79 μg which was comparable to vincristine with LC_{50} value of 0.33 μg. The compound did not exhibit any antimicrobial activity against Gram +ve and Gram −ve organisms, as well as against the tested fungal strains. But very good free radical scavenging activity was observed at concentration range of 0.185–100 μg with IC_{50} values of 1.43 μg in comparison to reference standard butylated hydroxytoluene (BHT) with IC_{50} value 16.46 μg.

1. Introduction

The chemistry of thiourea derivatives has received considerable attention in view of their variable bonding modes, promising biological implications. They have been used as drugs and are reported to possess a wide variety of biological activities against the bacteria and fungi. Thiosemicarbazones are compounds that have been studied for a considerable period of time for their biological properties. Thiosemicarbazone, a large group of thiourea derivatives, exhibits various biological activities and has therefore attracted considerable pharmaceutical interest [1]. They have been evaluated as antiviral [2], antioxidant [3], antibacterial [4, 5], antitumor [6, 7], anticancer [8, 9], and antitubercular [10–12] therapeutics over the last 50 years, whose biological activities are a function of parent aldehyde or ketone moiety [13]. Thiosemicarbazones are potent intermediates for the synthesis of pharmaceutical and bioactive materials, and, thus, they are used extensively in the field of medicinal chemistry [14]. A number of N-substituted arylthiosemicarbazide derivatives were prepared by the reaction of 2-hydrazinocarbonyl-3-chloro-5-phenoxy-benzo[b]thiophene with different substituted phenyl isothiocyanate, and the compounds were evaluated for antimicrobial and antitubercular activity [15]. A series of new derivatives of α-phenylcinnamoyl thiosemicarbazides have been synthesized and were screened for their antibacterial activity [16]. In view of the above applications, the present work was designed to synthesise N^1-phenylhydrazine-1,2-*bis*(carbothioamide) (Scheme 1) and was investigated for antibacterial, cytotoxic, and antioxidant activities.

2. Material and Methods

2.1. General. All chemicals used were of reagent grade (supplied by Sigma) and used as supplied. The FT IR spectra in the range (4000–400) cm^{-1} were recorded as KBr disc on FT IR 8300 Shimadzu Spectrophotometer. ^1H and ^{13}C



Scheme 1: Synthesis of N^1-phenylhydrazine-1,2-*bis*(carbothioamide).

NMR spectra were obtained on a Bruker AV-I console operating at 400 MHz. ^{1}H COSY experiments were executed using a 9.4 T Oxford magnet equipped with a Bruker AV-I console operating at 500 MHz. The UV-visible spectra were measured in DMSO using Shimadzu UV-Vis. 160A spectrophotometer in the range (200–1100) nm. Elemental microanalysis was carried out using CHN elemental analyzer model 5500-Carlo Erba instrument. Melting point of the compound was recorded and uncorrected.

2.2. Experimental

2.2.1. Synthesis of N^1-Phenylhydrazine-1,2-bis(carbothioamide). The thiosemicarbazide (0.9113 g, 10 mmol) was dissolved in hot ethanol (30 mL) and phenylisothiocyanate (1.3518 g, 10 mmol) was added slowly to this solution, then the mixture was refluxed at 100°C for two hours. A white powder was formed, cooled and filtered, washed with 50% cold ethanol, and dried in the desiccator. The compound was recrystallized from ethanol. mp: 173-174°C and yield: 73%. UV-Vis (nm): 305 and 315. FT IR (cm^{-1}): 3320 (terminal NH_2 group), 3065 (N–H, Ph–N), 2905 (C–H, as), 2830 (C–H, s), 2430 (S–H), 1615 (Ph ring), 1530 (N–C–N, as), 1420, 1120 (C–N), 1360 (Ph–N), and 1283 (C=S). ^{1}H-NMR (DMSO-d_6, δ in ppm, J in Hz): δ 9.66, 9.60, 9.36, 8.06, 7.49 (d, 2H, J = 8.6 Hz), 7.31 (t, 2H, J = 7.6; J = 8.6 Hz), 7.14 (t, 1H, J = 7.6 Hz). ^{13}C-NMR (DMSO-d_6, in ppm,): δ 187.4, 182.8, 141.5, 132.4, 131.0, and 129.5. Elemental analysis found % C 42.52 (42.42), H 4.58 (4.45), N 24.85 (24.74) calculated for $C_8H_{10}N_4S_2$.

2.2.2. Antimicrobial Screening. The disc diffusion method [17, 18] was used to test antimicrobial and antifungal strains against five Gram-positive (*Bacillus cereus*, *Bacillus megaterium*, *Bacillus subtilis*, *Staphylococcus aureus,* and *Sarcina lutea*), eight Gram-negative organisms (*Escherichia coli*, *Pseudomonas aeruginosa*, *Salmonella paratyphi*, *Salmonella typhi*, *Shigella boydii*, *Shigella dysenteriae*, *Vibrio mimicus,* and *Vibrio parahaemolyticus*), and three fungi (*Candida albicans*, *Aspergillus niger,* and *Saccharomyces cereviciae*). The bacterial and fungal strains used for the experiment were collected as pure culture from the Institute of Nutrition and Food Sciences (INFS), University of Dhaka. The test sample solutions containing the compound were made by dissolving in calculated volumes of solvents separately and applied to sterile discs (6 mm diameter) at a concentration of 200 and 400 μg/disc and carefully dried to evaporate the residual solvents. Discs containing the test compound were placed on nutrient agar medium uniformly seeded with the test microorganisms. Standard antibiotic Kanamycin (30 μg/disc) discs and blank discs (impregnated with solvents) were used as positive and negative controls, respectively. The antimicrobial activity of the test agent N^1-phenylhydrazine-1,2-*bis*(carbothioamide) was determined by measuring the diameter of zone of inhibition expressed in millimeter. The experiment was carried out in triplicate.

2.2.3. Antioxidant Activity. The antioxidant activity of the compound on the stable radical 1,1-diphenyl-2-picrylhydrazyl (DPPH) was determined by the method of Brand-Williams [19]. In the experiment, 2 mg of the compound, N^1-phenylhydrazine-1,2-*bis*(carbothioamide), was dissolved in methanol. Solution of varying concentrations such as 500, 250, 125, 62.50, 32.25, 15.625, 7.813, 3.906, 1.953, and 0.977 μg/mL were obtained by serial dilution technique. Two milliliters of a methanol solution of the extract of each concentration were mixed with 3 mL of a DPPH-methanol solution (20 mg/L) and allowed to stand for 30 min for the reaction. Then, the absorbance was measured at 517 nm using an Analytic Jene Spekel 1300 UV spectrophotometer, and from these values, the corresponding percentage of inhibitions was calculated by using the following equation:

$$\%\ \text{inhibition} = \left[1 - \left(\frac{\text{ABS sample}}{\text{ABS Control}}\right)\right] \times 100. \quad (1)$$

Then, percent inhibitions were plotted against respective concentrations. The IC control values were calculated as the concentration of each sample required to give 50% DPPH radical scavenging activity from the graph. The BHT (tert-butyl-1-hydroxytoluene) a potential antioxidant, was used as positive control. The experiment was performed in three replicates.

2.2.4. Brine Shrimp Lethality Bioassay. The brine shrimp lethality bioassay was used to predict the cytotoxic activity [20, 21] of the compound. For the experiment, 4 mg of the compound N^1-phenylhydrazine-1,2-*bis*(carbothioamide) was dissolved in dimethyl sulfoxide (DMSO) and solutions of varying concentrations (400, 200, 100, 50, 25, 12.5, 6.25, 3.13, 1.56, and 0.78 μg/mL) were obtained by the serial dilution technique using simulated seawater. The solutions were then added to the premarked vials containing 10 live brine shrimp nauplii in 5 mL simulated seawater. After 24 h, the vials were inspected using a magnifying glass, and

the number of survived nauplii in each vial was counted. The mortality endpoint of this bioassay was defined as the absence of controlled forward motion during 30 sec of observation [19]. From this data, the percent of lethality of the brine shrimp nauplii for each concentration and control was calculated. Vincristine sulphate was used as positive control.

3. Results and Discussion

The compound N^1-phenylhydrazine-1,2-*bis*(carbothioamide) was synthesised from phenylisothiocyanate reacting with thiosemicarbazide refluxing the mixture in ethanol for two hours with 73% yield (Scheme 1). The synthesised compound was characterised by elemental analysis, IR, UV-Vis, NMR spectroscopic methods. The compound showed maximum absorption (λ_{max}) at 305 and 315 in solvent DMSO. The IR spectra of N^1-phenylhydrazine-1,2-*bis*(carbothioamide) was ascertained by the presence of characteristic bands. A band appearing at 3320 cm^{-1} is assigned to terminal NH_2 group. The band at 1360–1420 cm^{-1} is due to C–N stretching vibration, while the band appearing at 1283 cm^{-1} is assigned to C=S stretching vibration.

In the NMR spectrum, the three most deshielded signals were broad. The chemical shifts of acidic protons of N–H are about δ 9.66, 9.60, 9.36 ppm. Signal at δ 8.06 ppm shows one signal due to the presence of one $-NH_2$ group (partially exchanged with D_2O present in DMSO-d_6). The other protons at benzene ring resonance come out in downfield, and this can be corroborated with concrete examples: The *ortho*-protons, δ = 7.49 ppm (d, 2H, J = 8.6 Hz), appear in downfield due to weak intramolecular hydrogen-bonded reaction N-H$\cdots$C. The *meta*-protons appear at δ = 7.31 ppm (t, 2H, J = 7.6; J = 8.6 Hz), the *para*-proton appears at δ = 7.14 ppm (t, 1H, J = 7.6 Hz).

The most deshielded ^{13}C-NMR signals correspond to C = S groups. The carbon atom of thiocarbonyl groups δ 182.8, 187.4 ppm shows the highest values, due to the lower excitation energy n-π^* and due to the existence of the intramolecular hydrogen bond related to the thionyl sulfur atom. This suggests that the use of very strong electron-withdrawing substituents may reduce the nucleophilic character of the C=S group.

The COSY spectrum reveals the ^{1}H–^{1}H coupling interactions in a molecule. It is usually plotted as three-dimensional contours, where the conventional spectrum is represented along the diagonal (Figure 1). The cross-peaks along both the sides of the diagonal identify the nuclei that are coupled to each other. On the contrary, the protons that are decoupled from the adjacent ones due to the lack of α-protons will show no correlation in the spectrum.

For instance, in the COSY spectrum of the compound, absence of any off diagonal peaks extending from δ = 9.66 to 8.06 ppm confirms their assignment to N–H protons. Based on the cross-correlation pattern of aromatic *ortho* (*o*), *meta* (*m*) *or, para* (*p*) protons of the compound, it is confirmed that the triplet (t) at 7.31 ppm (J = 8.6 Hz) corresponds to two *meta*-protons that showed two strong 1,2-correlations with neighboring *ortho*- and *para*-protons. The doublet (d) at 7.49 ppm (J = 8.6 Hz) corresponds to two *ortho*-protons that showed a weak 1,3 cross-correlation with the *para*-proton at 7.14 ppm (t, J = 7.6 Hz). So the electron density order looks $p > m > o$. But, theoretically the order should be $p > o > m$ *or* $p = o > m$. The thioamide-protons show no cross-correlation at all, because they are three or four bonds apart from each other.

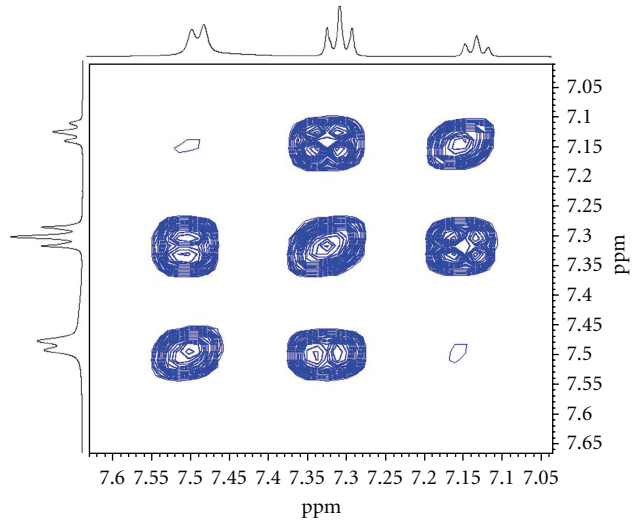


Figure 1: ^{1}H–^{1}H COSY spectrum.

3.1. Antimicrobial Screening. The compound did not exhibit any antimicrobial or antifungal activity against any organism tested.

3.2. Antioxidant Activity. The DPPH antioxidant assay is based on the ability of 1,1-diphenyl-2-picryl-hydrazyl (DPPH), a stable free radical, to be decolorized in the presence of antioxidants. The DPPH radical contains an odd electron, which is responsible for the absorbance at 517 nm and also for visible deep purple color. When DPPH accepts an electron donated by an antioxidant compound, the DPPH is decolorized which can be quantitatively measured from the changes in absorbance. The IC_{50} value of DPPH radicals scavenging for the compound was 6.44 ± 0.04 μg/mL, respectively, which is comparable to the reference standards *tert*-butyl-1-hydroxytoluene (BHT) (Table 1). In case of antioxidant screening, the compound showed very strong free radical scavenging activity (IC_{50} value of 1.43 μg/mL) and comparable to the reference standard BHT (IC_{50} value of 16.46 μg/mL).

3.3. Brine Shrimp Lethality Bioassay. In Brine shrimp lethality bioassay, the LC_{50} value of the compound was found to be 12.79 μg/mL (Table 2). However, varying degree of lethality to *Artesia salina* was observed with exposure to different dose levels of the test samples. In other words, % mortality increased gradually with the increase in concentration of

Table 1: Determination of IC_{50} values of the compound in DPPH.

Compound	Sl no.	Absorbance (Blank)	Concentration (μg/mL)	Absorbance	% Inhibition	IC_{50} (μg/mL)
Butylated hydroxytoluene (BHT)	1	0.378	500.000	0.025	94.709	16.46
	2	0.378	250.000	0.024	93.651	
	3	0.378	125.000	0.035	90.741	
	4	0.378	62.500	0.099	73.810	
	5	0.378	31.250	0.149	60.582	
	6	0.378	15.625	0.232	38.624	
	7	0.378	7.813	0.264	30.159	
	8	0.378	3.906	0.291	23.016	
	9	0.378	1.953	0.303	19.841	
	10	0.378	0.977	0.308	18.519	
N^1-Phenylhydrazine-1,2-*bis* (carbothioamide)	1	0.393	100.000	0.083	78.88	1.43
	2	0.393	50.000	0.084	78.62	
	3	0.393	25.000	0.090	77.09	
	4	0.393	12.500	0.096	75.57	
	5	0.393	6.250	0.104	73.54	
	6	0.393	3.125	0.111	71.75	
	7	0.393	1.563	0.111	71.75	
	8	0.393	0.781	0.226	42.49	
	9	0.393	0.391	0.253	35.62	
	10	0.393	0.185	0.368	6.36	

Table 2: Determination of LC_{50} values of the compound in Brine Shrimp Nauplii.

Compound	Conc. (μg/mL)	Log conc.	% mortality	Corrected %*	Probit	LC_{50} value from graph (μg/mL)
Vincristine sulfate	10	1.0	100	97.5	6.96	0.33
	5.000	0.698	90	90	6.28	
	2.500	0.397	80	80	5.84	
	1.250	0.096	80	80	5.84	
	0.625	−0.204	60	60	5.25	
	0.313	−0.505	50	50	5.00	
	0.156	−0.806	40	40	4.75	
	0.078	−1.107	20	20	4.16	
N^1-Phenylhydrazine-1,2-*bis* (carbothioamide)	200	2.301	100	97.5	6.96	12.79
	100	2.0	80	80	5.84	
	50	1.699	70	70	5.52	
	25	1.398	50	50	5.00	
	12.5	1.097	40	40	4.75	
	6.25	0.796	30	30	4.48	
	3.125	0.495	30	30	4.48	
	1.563	0.194	20	20	4.16	
	0.781	−0.107	10	10	3.72	

*Corrected: for the 100% mortality: $100[(n - 0.25)/n]$, where n = number of shrimps in each group.

the test samples. The compound was more toxic to brine shrimp than the reference standard, vincristine sulphate (LC_{50}: 0.34 μg/mL).

Brine shrimp lethality bioassay is a rapid and comprehensive bioassay for the bioactive compound of the natural and synthetic origin. By this method, synthesized pure compound can be tested for their bioactivity. In this method, *in vivo* lethality in a simple zoological organism (Brine shrimp nauplii) is used as a favourable monitor for screening and fractionation in the discovery of new bioactive compounds. This bioassay indicates cytotoxicity as well as a wide range of pharmacological activities such as antimicrobial, antiviral, pesticidal, and antitumor, and so forth. From the results of the brine shrimp lethality bioassay, it can be well predicted that the compound has considerable cytotoxic potency. Further bioactivity-guided investigation can be done to find out potent antitumor and pesticidal activity of the compound.

4. Conclusion

The results of the spectroscopic and elemental analyses were in good agreement with the structure of the proposed compound. It was found that the compound showed brine shrimp lethality with LC_{50} value of 12.79 μg which was comparable to vincristine with LC_{50} value of 0.33 μg. The compound did not exhibit any antimicrobial activity against Gram +ve and Gram −ve organism, as well as against the tested fungus. But very good free radical scavenging activity was observed at concentration range of 0.185–100 μg with IC_{50} values of 1.43 μg in comparison to reference standard butylated hydroxytoluene (BHT) with IC_{50} value 16.46 μg. Finally, from these bioassay studies report, the compound can be exploited as cytotoxic agent against tumor or cancer and as antioxidant for free radical scavenging.

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