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Short Note

2-{[(4-Hydroxy-3,5-dimethoxyphenyl)methylidene] hydrazinylidene}-4-oxo-1,3-thiazolidin-5-yl Acetic Acid

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Abstract: Thia-Michael addition of 2-[(4-hydroxy-3,5-dimethoxyphenyl)methylidene]hydrazine-1-carbothioamide (**1**) with maleic anhydride results in the formation of the title compound 2-{[(4-hydroxy-3,5-dimethoxyphenyl)methylidene]hydrazinylidene}-4-oxo-1,3-thiazolidin-5-yl acetic acid **2**. The precursor **1** is synthesized by the reaction of 4-hydroxy-3,5-dimethoxybenzaldehyde and thiosemicarbazide in the presence of glacial acetic acid as the catalyst. The structure of the title compound is determined by elemental analysis, FT-IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data. In order to determine the molecular interactions with the bacterial enzyme, the title compound is further docked into the active site of the MurB protein of *Staphylococcus aureus* (PDB ID: 1HSK). The in vitro antibacterial and antifungal activity of the title compound is carried out in order to appraise its antimicrobial efficacy by determination of zone of inhibition and minimal inhibitory concentration. The compound is also evaluated for its antioxidant property by 2,2-diphenyl-1-picryl-hydrazyl (DPPH) radical scavenging assay.

Keywords: Thia-Michael addition; maleic anhydride; in vitro antimicrobial evaluation; molecular docking; DPPH radical scavenging assay

1. Introduction

2-Hydrazolyl-4-thiazolidinones have emerged as a perceptible class of compounds, annexing thiosemicarbazones with 4-thiazolidinones, each with distinct biological activities. Thiosemicarbazones have been reported for their wide spectrum of antibacterial [1], anticancer [2,3], anti-inflammatory and antioxidant activities [4]. On the other hand, the chemical modification of compounds containing 4-thiazolidinone moiety leads to their wide appositeness as efficacious pharmacological agents, paving the way for their discernible antimicrobial [5,6], antimalarial [7], anti-HIV [8], antioxidant [9], anticancer [10–13], antiarrhythmic [14] and anti-inflammatory [15,16] activities. The array of biological responses exhibited by this coalescence of thiosemicarbazone with 4-thiazolidinone moiety has attracted researchers to further explore the potency of these compounds. One of the conducive synthetic methods for the preparation of 2-hydrazolyl-4-thiazolidinones includes thia-Michael addition reaction, employing maleic anhydride as the Michael acceptor [17–19].

Development of molecules with the property of targeting proteins concerned with initial stages of peptidoglycan biosynthesis can assure a better bactericidal effect than most of the clinically

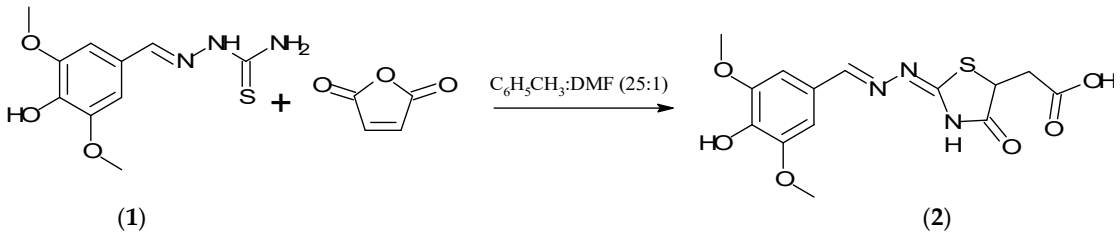
used antibiotics intending to destine towards the later stages. The imperative catalytic role of uridine diphosphate *N*-acetylenolpyruvylglucosamine reductase (MurB) in the bacterial peptidoglycan synthesis was attributed to the reduction of enolpyruvate moiety to D-lactate, to which a series of step-wise addition of the pentapeptide side chains occur. The absence of any homologue to MurB enzyme in eukaryotic cell and its involvement in the initial stage of peptidoglycan synthesis, makes it an ideal target for the development of bactericidal agents [20].

Molecular docking is a technique of simulation of molecular systems, which aids in the virtual screening and rational design of compounds to predict their bioactivities before being synthesized. Based on the reports which exhort the inhibition of MurB protein by 4-thiazolidinone derivatives [21,22], the title compound was docked into the active pocket of the enzyme. A literature survey and structure-activity relationship studies revealed the fact that the antibacterial property not only depended on the 4-thiazolidinone core, but also on the nature and positions of the substituents on the ring [23]. Thus, in view of eliciting the authentic pharmacological potency of the title compound, the in vitro microbial sensitivity assay has been carried out against the bacterial strains *Staphylococcus aureus*, *Bacillus subtilis*, *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, followed by *Aspergillus niger* and *Alternaria alternata* fungal strains.

Furthermore, the antioxidant assay of the title compound has been consummated to analyze its effectiveness as an antioxidant agent. The assay depends on the reduction of 2,2-diphenyl-1-picryl-hydrazyl (DPPH), which gives a strong absorption maxima at 517 nm (purple colour) due to the odd electron, which in the presence of a hydrogen donor in the compound, undergoes pairing up, resulting in the curtailed absorption intensity.

2. Results

The schematic pathway for the preparation of the title compound is outlined in Scheme 1. The title compound was obtained via thia-Michael addition reaction between maleic anhydride employed as a Michael acceptor and 2-[(4-hydroxy-3,5-dimethoxyphenyl)methylidene] hydrazine-1-carbothioamide **1**, in a mixture of toluene and DMF (25:1). The compound **1** was in turn synthesized through the hydrazone condensation between 4-hydroxy-3,5-dimethoxybenzaldehyde and thiosemicarbazide in ethanol, using a few drops of acetic acid as the catalyst [17]. The structure and purity of compound **2** was ascertained by FT-IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data (See the Supplementary Materials).



(**1**) (**2**)

Scheme 1. Synthesis of 2-{[(4-hydroxy-3,5-dimethoxyphenyl)methylidene]hydrazinylidene}-4-oxo-1,3-thiazolidin-5-yl acetic acid.

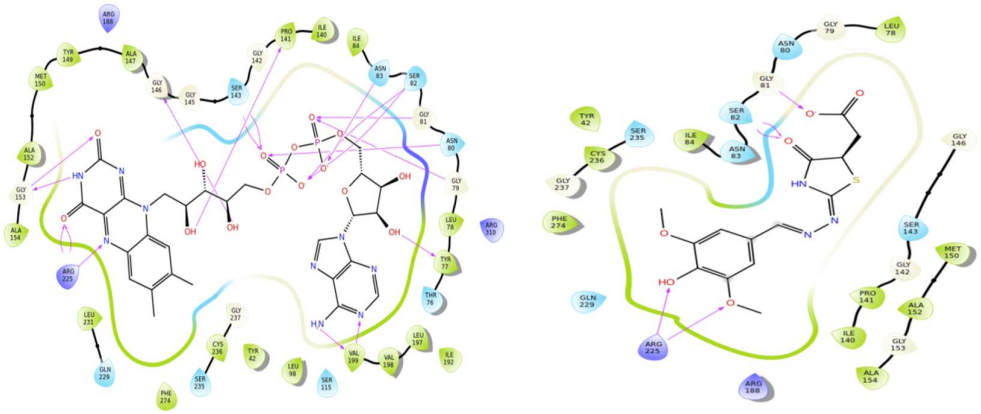
2.1. Molecular Docking

In view of discerning the binding affinity, binding energy and orientation of ligand-enzyme interactions, the title compound was docked into the active pocket of the MurB enzyme. The docked title compound displayed hydrogen-bonding interactions with the amino acid residues in the active site of the enzyme. The comparison of the docking score between the title compound and the bound ligand, FAD (Flavin-Adenine dinucleotide) is elucidated in Table 1, followed by Figure 1, which depicts the 2D docking poses of FAD (Figure 1a) and the title compound (S-isomer) (Figure 1b) in the active site of MurB enzyme. It was observed that an analogous type of molecule, [Compound **3**] with an additional phenyl group attached to the nitrogen atom of the thiazolidinone ring and lacking

one methoxy substituent at the fifth position of the phenyl ring, did not exhibit any activity against the tested bacterial strains [24]. Thus, a correlative docking analysis was carried out between the title compound and compound **3** for the comparative analysis of the molecular interactions with the bacterial enzyme. The docking score of compound **3** is expounded in Table 2 followed by Figure 2, which exemplifies its binding interactions with the MurB enzyme.

Table 1. Docking score of the compound **2** and the bound ligand FAD.

Ligand	Docking Score (kcal/mol)
Compound **2**	−8.374
FAD	−8.041



(**a**) (**b**)

Figure 1. 2D docking pose of (**a**) FAD and (**b**) Compound **2** in the active site of *Staphylococcus aureus* MurB enzyme.

Table 2. Docking score of the compound **3**.

Ligand	Docking Score(kcal/mol)
Compound **3**	−4.726

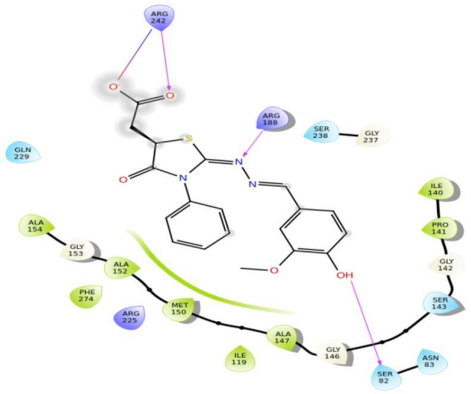


Figure 2. 2D docking pose of Compound **3** in the active site of *Staphylococcus aureus* MurB enzyme.

2.2. In Vitro Antimicrobial Assay

The title compound was screened for its antimicrobial activity against two Gram-positive (*Staphylococcus aureus* MTCC-7443, *Bacillus subtilis* MTCC-441) and two Gram-negative

(*Pseudomonas aeruginosa* MTCC-424, *Klebsiella pneumoniae* MTCC-139) bacterial strains, followed by two fungal species, namely *Aspergillus niger* MTCC-281 and *Alternaria alternata* MTCC-149. Tetracyclin was used as the standard antibiotic for antibacterial activity, whereas Nystatin was employed for antifungal evaluation. The values of the diameter of inhibition zone (mm) are tabulated in Tables 3 and 4, depicting the results of the antibacterial and antifungal evaluation for the title compound with respect to the standard.

Table 3. Result of antibacterial activity for compound **2** with respect to the standard (Disc diffusion method).

Zone of Inhibition (mm)				
Compound	***Staphylococcus aureus***	***Bacillus subtilis***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
2	20.0	22.0	17.5	18.0
Tetracycline	26.0	22.0	25.0	23.0

Table 4. Result of antifungal activity for compound **2** with respect to the standard (Disc diffusion method).

Zone of Inhibition (mm)		
Compound	***Aspergillus niger***	***Alternaria alternata***
2	20.0	15.5
Nystatin	25.0	22.0

In conjunction with the disc diffusion method applied for the antimicrobial evaluation, the parallel determination of minimum inhibitory concentration of the sample extract was accomplished with different concentration of extract in Mueller-Hinton broth for bacteria and potato-dextrose broth for fungi by using macro dilution method. The minimum inhibitory concentration (MIC) values for the compound **2** with reference to the standard antibiotic, for the antibacterial and antifungal evaluation are explicated in Tables 5 and 6 respectively.

Table 5. MIC value of compound **2** with respect to the standard (Antibacterial evaluation).

Minimum Inhibitory Concentration (μM)				
Compound	***Staphylococcus aureus***	***Bacillus subtilis***	***Klebsiella pneumoniae***	***Pseudomonas aeruginosa***
2	14.2	7.1	56.6	42.5
Tetracycline	6.3	8.1	12.4	14.9

Table 6. MIC value of compound **2** with respect to the standard (Antifungal evaluation).

Minimum Inhibitory Concentration (μM)		
Compound	***Aspergillus niger***	***Alternaria alternata***
2	14.2	70.8
Nystatin	4.9	6.7

2.3. In Vitro Antioxidant Activity (DPPH Radical Scavenging Assay)

The title compound was screened for its in vitro antioxidant property by DPPH radical scavenging assay using ascorbic acid as the standard. The effectiveness of the compound to scavenge the DPPH radical was calculated using Equation (1):

$$\text{DPPH scavenging effect (\%)} = (A_0 - A_1/A_0) \times 100 \quad (1)$$

where, A_0 is the absorbance of the control reaction and A_1 is the absorbance in the presence of the compound **2** or ascorbic acid. Table 7 depicts the result of the in vitro antioxidant assay of the compound **2** with respect to the standard.

Table 7. Result of in vitro antioxidant activity of the compound **2** with reference to the standard.

Compound	Concentration (μM)	% DPPH Scavenging
2	283	68.19 ± 0.59
Ascorbic acid	567	94.58 ± 0.75

3. Discussion

The FT-IR spectrum of the title compound exhibited absorption band at 1645 cm^{-1} corresponding to the C=O stretching due to the carboxylic acid group. The absorption band observed at 1711 cm^{-1} characteristic of carbonyl stretching incorporated in the lactam ring justifies the existence of the title compound in the lactam tautomeric form. The absorption bands at 3436 cm^{-1} and 3363 cm^{-1} account for the N-H and O-H stretching. The C-O and C-N stretching resulted in the absorption bands at 1187 cm^{-1} and 1321 cm^{-1} respectively. Two distinct bands observed at 1270 cm^{-1} and 1002 cm^{-1} exemplifies the asymmetric and symmetric stretching vibrations of the =C-O-C group of the methoxy substituents. The forked absorption band at 2965 cm^{-1} justifies the aliphatic C-H stretching vibration, whereas the one at 1615 cm^{-1} expounds the presence of C=N stretch. Furthermore, the C-S stretching vibration is delineated by an absorption band at 625 cm^{-1}.

The ^{1}H-NMR spectrum of the compound exhibited distinctive system of AMX pattern due to 4-thiazolidinone moiety. The two diastereotopic methylene protons H_A and H_M displayed two doublet of doublets at 2.85 ppm (J_{AM} =17.6 Hz, J_{AX} =8.4 Hz) and 2.98 ppm (J_{MA} =17.6 Hz, J_{MX} =3.2 Hz) respectively, whereas the doublet of doublet discerned at 4.32 ppm was conferred to the proton H_X of the thiazolidinone ring, with J_{XA} = 8.4 Hz and J_{XM} = 3.2 Hz. The six equivalent protons of the two methoxy substituents of the phenyl ring resonated at 3.80 ppm, whereas the two aromatic protons of the phenyl ring resonated at 7.04 ppm. The two singlets observed at 8.26 ppm and 8.99 ppm were ascribed to the azomethine proton and the proton of the hydroxyl substituent respectively. The -NH proton of the ring and the carboxyl proton resonated as a broad singlet at 12.14 ppm.

The ^{13}C-NMR spectrum of the title compound displayed signals at 175.9 and 172.2 ppm accounting for the carbon of the carboxylic group and the carbonyl carbon of the lactam ring respectively. The CH=N carbon of the azomethine group resonated at 157.0 ppm, whereas the presence of C=N carbon, resulted in a signal at 162.9 ppm. The two carbons of the $–OCH_3$ substituents of the phenyl ring resonated at 56.4 ppm. The signal for the carbon of the $–CH_2$ group appeared at 37.2 ppm, whereas the lactam ring carbon attached to it resonated at 44.0 ppm. The molecular weight of the compound was confirmed by the mass spectrum of the compound, which gave the molecular ion peak m/z at 354.0 (M^+ + 1), which concurred with the molecular formula $C_{14}H_{15}N_3O_6S$. However, no attempt was made to separate the possible optical isomers, which are destined to be formed due to the presence of the chiral center.

Molecular docking results of the stable tautomer of the title compound showed hydrogen bonding interactions between the title compound and the amino acid residues constituting the active site of the MurB enzyme. Both the optical isomers of the title compound exhibited distinct hydrogen bonding interactions involving Ser 82 residue with the carbonyl oxygen of the lactam ring as well as between the oxygen of the carboxylate moiety of the title compound and Gly 81 residue, with the S isomer displaying a better docking score as compared to the R isomer. In addition to these interactions, the oxygen of the –OH and one of the $–OCH_3$ groups attached to the phenyl ring displayed hydrogen bonding with the Arg 225 residue of the enzyme. Compound **3** displayed one salt-bridge and three hydrogen bonding interactions with the amino acid residues in the active pocket of the MurB enzyme. The drastic difference in the docking scores of the compound **3** and the title compound justifies the

experimentally observed antibacterial potency of compound **2** with respect to compound **3** which is inactive against the tested bacteria. Furthermore, the presence of an extra methoxy group in the title compound aids in its better interaction with the target enzyme, making it a more efficient antibacterial agent.

The title compound exhibited antibacterial activity with distinctive efficacy as revealed by the values of the diameter of inhibition zone and MIC. The results of the disc diffusion method not only showed a broad spectrum of antibacterial activity of the compound against both Gram positive and Gram negative bacterial strains, but also proved it to be equally efficient as that of the standard drug tetracycline against *Bacillus subtilis*. Furthermore, the MIC values affirmed the moderate antibacterial efficacy of the compound against the tested bacterial strains and its exalted efficacy against *Bacillus subtilis*. The compound showed growth inhibitory effect on being subjected to antifungal assay at the tested concentration. A conjoint evaluation of the results of disc diffusion method and MIC, corroborated the augmented efficacy of the compound as an antifungal agent against *Aspergillus niger*, as compared to *Alternaria alternata*.

The result of the DPPH radical scavenging assay showed the moderate activity of the title compound as an antioxidant agent, with reference to ascorbic acid taken as the standard.

4. Materials and Methods

All reagents and chemicals were purchased from Sigma-Aldrich India (Bangalore, India) and used without further purification. The melting point of the title compound was determined using an open capillary tube (Shiv Scientific Stores (Regd.), Delhi, India) and was uncorrected. The reaction completion and purity of the reaction products were monitored by thin layer chromatography using Merck (Darmstadt, Germany) silica gel 60 F_{254} coated aluminum plates. FT-IR spectrum was recorded on Bruker-FTIR Infrared spectrometer (Bruker, Billerica, Massachusetts, United States) (υ_{max} in cm^{-1}). ^{1}H-NMR (400 MHz) spectrum was recorded on a Bruker Avance III HD Nanobay 400 MHz FT-NMR spectrometer (Buker Biospin AG, Fällanden, Switzerland), with 5 mm multinuclear probes, whereas the ^{13}C-NMR (100 MHz) spectrum was recorded in DMSO (Dimethyl sulphoxide) solvent at 100 MHz with tetramethylsilane (TMS) as the internal standard. Mass spectrum was obtained using Shimadzu LCMS-8030 mass spectrometer (Shimadzu, Kyoto, Japan). Elemental analyses data was accomplished using VARIO EL-III (Elemental AnalysensystemeGmBH, Langenselbold, Germany).

Schrödinger Software Suite 2015-2 (Schrödinger, New York, NY, USA) was the software employed for docking studies, with hardware 2x Intel Xeon 1.9 GHz E5-2420/6C/15MB Cache RAM 6 × 4 Gb DDR-3 1333 MHz ECC RDIMM 4 × 500 Gb Graphics Card NvidiaQuadro 600 machine. The microorganisms used in the antimicrobial assay were collected from Institute of Microbial Technology (IMTECH), (Chandigarh, India). Sterile empty discs (6.0 mm) were procured from Himedia Company, (Mumbai, India). The absorbance measurement in antioxidant assay was performed using Systronics Spectrophotometer 106, with the wavelength range of 340 nm to 960 nm (Systronics, Ahmedabad, India).

4.1. Synthetic Protocol of 2-{[(4-hydroxy-3,5-dimethoxyphenyl)methylidene]hydrazinylidene}-4-oxo-1,3-thiazolidin-5-yl acetic Acid

2-[(4-Hydroxy-3,5-dimethoxyphenyl)methylidene] hydrazine-1-carbothioamide(**1**) (255 mg, 1 mmol) was prepared in accordance with the reported experimental protocol [17]. Compound **1** was then subjected to Thia-Michael addition with maleic anhydride (98 mg, 1 mmol) in a mixture of 12.5 mL toluene and 0.5 mL *N*,*N*-dimethyl formamide (DMF). The reaction mixture was refluxed for 8 h and the solid obtained was filtered, washed with toluene, dried and recrystallized using DMF to obtain the title compound as a white colored solid with a yield of 79% (278 mg).

Melting point: 282–284°C; MS: m/z = 354.0 (M^+ + 1); FT-IR: υ_{max} (cm^{-1}), 3436 (N-H), 3363 (O-H), 2965 (Al-H), 1711 (lactam ring C=O), 1645 (carboxylic C=O), 1615 (C=N), 1321 (C-N), 1270 (=C-O-C, asymmetric stretch), 1187 (C-O), 1002 (=C-O-C, symmetric stretch), 625 (C-S); ^{1}H-NMR (400 MHz, DMSO-d_6): ppm,

2.85 (dd, 1H, H_A, J_{AM} = 17.6 Hz, J_{AX} = 8.4 Hz), 2.98 (dd, 1H, H_M, J_{MA} = 17.6 Hz, J_{MX} = 3.2 Hz), 3.80 (s, 6H, -OCH_3), 4.32 (dd, 1H, H_X, J_{XA} = 8.4 Hz, J_{XM} = 3.2 Hz), 7.04 (s, 2H, Ar-H), 8.26 (s, 1H, -CH=N-), 8.99 (s, 1H, -OH), 12.14 (broad s, 2H, -NH, -COOH); ^{13}C-NMR (100 MHz, DMSO-d_6): ppm, 37.2 (-CH_2), 44.0 (-CH), 56.4 (-OCH_3), 105.7, 124.9, 138.9, 148.5, 157.0 (HC=N-), 162.9 (C=N), 172.2 (lactam C=O), 175.9 (COOH); Elemental analysis: Calculated for $C_{14}H_{15}N_3O_6S$, C, 47.59%; H, 4.28%; N, 11.89%. Found: C, 47.53%; H, 4.26%, N, 11.81%.

4.2. Molecular Docking of Title Compound against Staphylococcus aureus MurB Enzyme

The docking of the title compound against the *Staphylococcus aureus* MurB protein was carried out using Schrödinger Glide. Schrödinger maestro 10.2 was utilized to draw the structure of the title compound using the provision of the 2D sketcher. The structure was then converted into 3D through its optimization using LigPrep. A 3D structure of the FAD-bound MurB protein was retrieved from the protein data bank (PDB ID: 1HSK), after which it was refined to add the missing hydrogen atoms, residues and amino acid side chains. The optimization of the protein was followed by minimization which was intended to avoid steric clashes. Glide software was then utilized to generate the receptor grid for the protein, which was followed by ligand docking using extra precession (XP) mode.

4.3. In Vitro Antimicrobial Assay (Disc Diffusion Method)

The bacterial strains were inoculated on Mueller-Hinton Agar (MHA), followed by the incubation for 24 h at 37°C. Consecutively, the fungal strains were inoculated on Potato Dextrose Agar (PDA) and then incubated for 48 h at 30°C, followed by the preparation of its suspension in saline solution (0.85% NaCl). 100 µL of the DMSO extract stock solution prepared by dissolving 50 mg of the title compound in 5 mL DMSO was loaded on sterile empty discs individually and aseptically, and then used for conducting antimicrobial assay according to the reported method [25].

For antibacterial assay, 200 µL of overnight grown bacterial culture was dispensed into 20 mL sterile nutrient broth followed by its incubation for 4–5 h at 37 °C in order to standardize the culture to 10^{-5} CFU/mL. 0.1 mL of the 24 h old bacterial culture was placed on MHA medium and spread all over on the plate using the spread plate technique. The PDA plates for fungi were analogously inoculated with a lawn of the test fungi. Sterile discs saturated with 100 µL of test solution were placed on MHA plate for bacteria and PDA plate for fungi after drying the discs under laminar air flow. The plates were incubated for 18–24 h at 37°C for bacteria, whereas the incubation was accustomed to 48 h at 30°C for fungi. Discs treated only with 100 µL DMSO without the sample, were used as negative controls, whereas tetracycline and nystatin discs were used as the positive controls for bacteria and fungi respectively. The clear, distinct, measurable circular zones of inhibition around the discs were used as an implication for antimicrobial activity.

4.4. In Vitro Minimum Inhibitory Concentration (MIC)

Minimum inhibitory concentration of the sample extract was determined by macro dilution method [26]. The 24 h old fresh bacterial strains were diluted 100 folds in MHB (100 µL of bacterial cultures in 10 mL MHB). Stock solution of the compound was prepared by dissolving 10 mg in 1 mL of DMSO. The test samples were added in increasing concentrations (1.25, 2.5, 5, 10, 20, ... up to 200 µL of stock solution containing 0.0125, 0.025, 0.050, 0.10, 0.20, ... up to 2.0 mg of the extract) to the test tubes containing bacterial cultures, followed by its incubation at 37°C for 24 h. A similar procedure was followed for fungal strains, in which the 48 h old culture of the fungal strains was diluted 100 folds in PDA, which is used as a diluent. The addition of different concentrations of stock solution was made according to the method described above and the tubes were incubated at 28°C for 48 h. The tubes were examined for visible turbidity using MHB and PDA as control. The lowest concentration that inhibited visible growth of test microbes was recorded as MIC.

4.5. In Vitro Antioxidant Activity (DPPH Radical Scavenging Assay)

Free radical scavenging activity of the title compound was determined using the reported method [27]. 1 mL of 1mM DPPH solution in ethanol was added to sample and standard solutions of 0.1 mg/mL concentration. The mixture was shaken well and then allowed to stand at room temperature in the dark for 30 min and the absorbance was measured at 517 nm using a UV-VIS spectrophotometer. The experiment was carried out in triplicate and the radical scavenging capacity was calculated using Equation (1).

5. Conclusions

A simple and straight-forward method for the synthesis of 4-thiazolidinone derivative is construed in the present study along with the structural confirmation of the compound using spectral data. The protein-ligand interaction study of the compound with MurB has been annexed along with its validation by conducting in vitro antimicrobial assay. The antibacterial evaluation displayed augmented activity against *Bacillus subtilis*, which was substantiated by the values of the disc diffusion assay and minimum inhibitory concentration values which prompts the efficiency of the compound to be developed as an antibacterial drug. The compound also exhibited a pronounced activity against *Aspergillus niger* among the tested fungal strains. Furthermore, the DPPH radical scavenging assay result illustrates the moderate antioxidant activity of the compound.

Supplementary Materials: FT-IR, ^{1}H-NMR, ^{13}C-NMR and mass spectral data are available online.

Author Contributions: S.K. performed the experiments, docking studies and antioxidant assay; S.C.K. conducted the antimicrobial evaluation; B.K.S. analysed the data; B.N. guided throughout the research work.

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Conflicts of Interest: The authors declare no conflicts of interest.

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