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A detailed point-by-point response to the reviewers' comments

We would like to thank the reviewers for the comments and endorsements of our work. Attached below is our detailed response to the comments on our manuscript. We have attached a revised version of the paper that addresses the issues raised by the reviewers. The revised parts in the manuscript are indicated by red characters.

Reviewer #3:

Comment:

1. The paper need more attention from the side of the authors.

- The abstract is somehow unorganized. Even if it is an unstructured abstract, it should respond to the following point one after the other. Background, aims, methods, results, and conclusion

- The background is missing.

- The aims are not clearly presented.

- Methods should be written in brief before presenting the results.

- Results they took the most of your abstract. Please be brief; most of the text is not important for a reader who only wants to get an idea about your paper before reading it. Just put your key results in brief.

- The conclusion is missing.

Response:

We appreciate the recommendation and rewrite the abstract as follows,

Polytopic organic ligands with hydrazone moiety are in the forefront of new drug research among many others due to their unique and versatile functionality and ease of strategic ligand design. Quantum chemical calculations of these polyfunctional ligands can be carried out *in silico* to determine the thermodynamic parameters. In this report two new tritopic dihydrazide ligands, N'2, N'6-bis[(1*E*)-1-(thiophen-2-yl) ethylidene] pyridine-2, 6-dicarbohydrazide (L1) and N'2, N'6-bis[(1*E*)-1-(1H-pyrrol-2-yl) ethylidene] pyridine-2, 6-dicarbohydrazide (L2) were successfully prepared by the condensation reaction of pyridine-2, 6-dicarboxylic hydrazide with 2-acetylthiophene and 2-acetylpyrrole**.** The FT-IR, ^{1}H and ^{13}C NMR as well as mass spectra of both L1 and L2 were recorded and analyzed. Quantum chemical calculations were performed at DFT/B3LYP/cc-pvdz/6-311+ G (d, p) level of theory to study the molecular geometry, vibrational frequencies, and thermodynamic properties including changes of $\Delta \boldsymbol{H}$, $\Delta \boldsymbol{S}$, and $\Delta \boldsymbol{G}$ for both the ligands. The optimized vibrational frequency and (^{1}H and ^{13}C) NMR obtained by B3LYP/cc-pvdz/6-311 + G (d, p) showed good agreement with experimental FT-IR and NMR data. Frontier molecular orbital (FMO) calculations were also conducted to find the HOMO, LUMO, and HOMO–LUMO gaps of the two synthesized compounds. To investigate the biological activities of the ligands, L1 and L2 were tested *in vitro* bioassays against some Gram-negative and Gram-positive bacteria and the fungus strain. In addition, Molecular docking was used to study the molecular behavior of L1 and L2 against tyrosinase from *Bacillus megaterium.* The outcomes revealed that both L1 and L2 can suppress microbial growth of bacteria and fungi with variable potency. The antibacterial activity results demonstrated the compound L2 to be potentially effective against *Bacillus Megaterium* with inhibition zones of 12 mm while molecular docking study showed the binding energies for L1 and L2 to be −7.7 and −8.8 kcal mol^{-1} respectively with tyrosinase from *Bacillus megaterium.*

- Font of the reference is different from the text

Response:

We appreciate the recommendation and corrected the font of the references.

- This sentence “Polytopic ligands containing hydrazide-hydrazone moiety (—CO—NHN=CH—) are important for new drug development” should be better connected to the next one to explain why they are important for new drug development?

Response:

We appreciate the recommendation and corrected the section as below:

Polytopic ligands containing hydrazide-hydrazone moiety (—CO—NHN=CH—) are important for new drug development [1-6]. Because, their polyfunctional nature offer multifarious synthetic ways to derivatize such organic molecules towards suitable and effective drug-receptor interaction. The derivatives of hydrazide-hydrazone moiety specially with heterocyclic system possess a range of biological activities namely, anti-microbial, anti-mycobacterial, antitubercular, anticonvulsant, anticholinesterase [1], antiplatelet, and more importantly antitumor [5,7]. Transition metal complexes derived from such type of ligands have been widely studied since they also demonstrate significant biological and pharmacological properties [8-11].

- Replace "In addition to that, the compounds were tested in vitro bioassays against some Gram-negative and Gram-positive bacteria and the fungus strain showing specially promising results for L2. Molecular docking methodology was used to study molecular behavior of L1 and L2 with *Bacillus Megaterium* to identify their binding interactions" with "In addition to that, the compounds were tested in vitro against Gram-negative and positive bacteria and two fungi. Molecular docking was used to study the molecular behavior of L1 and L2 against tyrosinase from *Bacillus megaterium*.

Response:

We appreciate the recommendation and corrected the section as below:

This Section is corrected as "In addition to that, the compounds were tested in vitro against Gram-negative and positive bacteria and two fungi. Molecular docking was used to study the molecular behavior of L1 and L2 against tyrosinase from *Bacillus megaterium*."

Section 2.3.1.

Reference for the used strains is required. Why are those specific strains used?

Response:

We appreciate the recommendation and added the required references as below.

We have these specimens of strains available in our lab to study. Therefore, we used. We also updated the standard ciprofloxacin and miconazole with Ceftriaxone and Amphotericin-B and corrected the reference the number of *Salmonella Typhi (K-323130)* bacteria. We are showing our sincere apology for such mistakes. The changes are added as follows,

2.1.3. Antimicrobial activity assay

In vitro antimicrobial activity of synthesized ligands was evaluated by agar disc diffusion method [40]. Mueller Hinton Agar (MHA) media (HIMEDIA, India) was used as a control medium for testing against bacteria and Potato Dextrose Agar (PDA) media (HIMEDIA, India) was used for fungal strain. After preparation, the MHA and PDA medias were incubated for 24 h and contaminations were checked. After incubation, the test organism was inoculated using sterile cotton bar on media. The sample discs were put gently on pre-inoculated agar plates and aerobically incubated for 24 h at 37 °C for antibacterial and for 48 h at 26 °C for antifungal assay. Dimethyl sulfoxide (DMSO) was used as control. Each disc was loaded with 25 µL of sample solution in DMSO containing 300 µg of synthesized compounds. 10 µL of ceftriaxone and amphotericin-B solutions containing 50 µg each in DMSO were loaded per disc for antibacterial and antifungal assays as positive control, respectively. The diameter of the inhibition zones in mm circling the disc were measured. Two gram-positive *Staphylococcus aureus* (cars-2) and *Bacillus Megaterium (BTCC-18)*, two gram-negative *Escherichia coli* (carsgn-2) and *Salmonella Typhi (K-323130)* bacteria, and two fungal strains *Trichoderma harzianum* (carsm-2) and *Aspergillus niger* (carsm-3) were used in this study.

Section 3.2. and 4.9.

PDB ID 4j6u is for **tyrosinase from Bacillus megaterium, not the bacillus megatherium crystal structure. Please change the info accordingly.**

Response:

We appreciate the recommendation, and corrected this information as shown below:

3.2. Protein-ligand Docking

3.2.1. Ligand and Protein preparation:

The structures of L1 and L2 have been fully optimized by using Gaussian 09 software at B3LYP/6-311G+ (d, p) level. The 3D crystal structure of tyrosinase from *Bacillus Megaterium* (PDB ID: 4j6u; resolution: 2.5Å, Chain A, B) was obtained in pdb format from online RCSB protein data bank (PDB) database. The structure was verified, and an energy minimization was performed with the Swiss-Pdb Viewer software packages (version 4.1.0) [44], since the crystal structure contains a variety of issues related to improper bond order, side chains geometry, and missing hydrogen atoms. Prior to docking, all the heteroatoms and water molecules were removed from the crystal structure using PyMol (version 1.3) software packages [45]. The active binding pocket of tyrosinase was predicted by CASTp—having the highest pocket area and volume are 95.432 $Å^2$ and 137.877 $Å^3$, respectively [46]. The binding site residues predicted by CASTp for tyrosinase were used for grid generation. Both the structures of the proteins and ligands were saved in .pdbqt format by AutoDock Vina (version 1.1.2, May 11, 2011) for docking analysis [47].

3.2.2. Molecular docking Analysis:

The docking calculations were performed using default parameters and 8 docked conformations were generated for both compounds. The energy calculations were done by genetic algorithms. Nonpolar hydrogen atoms, Gasteiger partial charges, rotatable bonds, and grid box with dimensions 66.57 × 58.25 × 84.98 $Å^3$ created on the tyrosinase with the aid of Auto Dock Tools 1.1.2 and spacing of 0.3750 Å. The docked conformation of the respective protein conformer with lowest binding free energy and root mean-square deviation value (RMSD) 0.0 Å was analyzed using PyMOL Molecular Graphics System (version 1.7.4) and Accelrys Discovery Studio 4.1 [49].

4.9. *Molecular Docking Study*

Molecular docking is a powerful tool to investigate and provide a proper understanding for ligand receptor interactions in order to facilitate the design of potential drugs [93-96]. To investigate and compare the antimicrobial activity of the synthesized compounds, docking analysis of L1 and L2 against tyrosinase from *Bacillus Megaterium* were performed. It is well-known that tyrosinase of *Bacillus Megaterium* bacteria is an attractive target for the development of antimicrobials or antibiotic adjuvants for the treatment of hyperpigmentation because of its similarity (33.5%) to the human enzyme [97-100].

Section 3.2. The data at least should be split into 2 more subheads, one for the ligand and receptor preparation and the second for the docking analysis. Divide the data into those 2 subheads and add more about the software used to perform the docking and the parameters used (Grid box, extensiveness …)

Response:

We appreciate the recommendation, and splitted the data into 2 more subheads as, 3.2.1. Ligand and Protein preparation and 3.2.2. Molecular docking analysis. Also, more information was added about the software used as shown below:

3.2. Protein-ligand Docking

3.2.1. Ligand and Protein preparation:

The structures of L1 and L2 have been fully optimized by using Gaussian 09 software at B3LYP/6-311G+ (d, p) level. The 3D crystal structure of tyrosinase from *Bacillus Megaterium* (PDB ID: 4j6u; resolution: 2.5Å, Chain A, B) was obtained in pdb format from online RCSB protein data bank (PDB) database. The structure was verified, and an energy minimization was performed with the Swiss-Pdb Viewer software packages (version 4.1.0) [44], since the crystal structure contains a variety of issues related to improper bond order, side chains geometry, and missing hydrogen atoms. Prior to docking, all the heteroatoms and water molecules were removed from the crystal structure using PyMol (version 1.3) software packages [45]. The active binding pocket of tyrosinase was predicted by CASTp—having the highest pocket area and volume are 95.432 $Å^2$ and 137.877 $Å^3$, respectively [46]. The binding site residues predicted by CastP for tyrosinase were used for grid generation. Both the structures of the proteins and ligands were saved in .pdbqt format by AutoDock Vina (version 1.1.2, May 11, 2011) for docking analysis [47].

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Section 4.8. There is no proper description of the results and no discussion in this part.

Please revise - This sentence should be moved to the materials and methods or deleted from the section "Two gram-positive Staphylococcus aureus (cars-2) and Bacillus Megaterium (BTCC-18), two gram-negative Escherichia coli (carsgn-2) and Salmonella Typhi (JCM-1652) bacteria, and two fungal strains Trichoderma harzianum (carsm-2) and Aspergillus niger (carsm-3) were used in this study."

Response:

We appreciate the recommendation, and this section is updated by changing the standard ciprofloxacin and miconazole with Ceftriaxone and Amphotericin-B. Also, the sentences of second part (4.8) are deleted and added to 2.3.1 as suggested by the reviewer.

2.1.3. Antimicrobial activity assay

In vitro antimicrobial activity of synthesized ligands was evaluated by agar disc diffusion method [40]. Mueller Hinton Agar (MHA) media (HIMEDIA, India) was used as a control medium for testing against bacteria and Potato Dextrose Agar (PDA) media (HIMEDIA, India) was used for fungal strain. After preparation, the MHA and PDA medias were incubated for 24 h and contaminations were checked. After incubation, the test organism was inoculated using sterile cotton bar on media. The sample discs were put gently on pre-inoculated agar plates and aerobically incubated for 24 h at 37 °C for antibacterial and for 48 h at 26 °C for antifungal assay. Dimethyl sulfoxide (DMSO) was used as control. Each disc was loaded with 25 µL of sample solution in DMSO containing 300 µg of synthesized compounds. 10 µL of ceftriaxone and amphotericin-B solutions containing 50 µg each in DMSO were loaded per disc for antibacterial and antifungal assays as positive control, respectively. The diameter of the inhibition zones in mm circling the disc were measured. Two gram-positive *Staphylococcus aureus* (cars-2) and *Bacillus Megaterium (BTCC-18)*, two gram-negative *Escherichia coli* (carsgn-2) and *Salmonella Typhi* (*K-323130*) bacteria, and two fungal strains *Trichoderma harzianum* (carsm-2) and *Aspergillus niger* (carsm-3) were used in this study.

4.8. Antimicrobial activity using agar disc diffusion method

In vitro sensitivities of two gram-positive and two gram-negative bacteria including two fungal strains against the synthesized compounds were evaluated by agar disc diffusion method. The

formation of diameter of inhibition zones in mm by the synthesized analogues are shown in **Table 5**. Compound L2 showed moderate activity against *Bacillus Megaterium* bacteria while L1 showed promising antifungal activity against *Aspergillus niger* fungal strains compared to standard Amphotericin-B.

Table 5. Diameter of inhibition zones (mm) of the synthesized compounds, Ceftriaxone and Amphotericin-B against tested bacterial and fungal strains.

Compd.	Gram (+) bacteria		Gram (-) bacteria		Fungi	
	S. aureus	*B. megaterium*	*E. coli*	*S. typhi*	*T. harzianum*	*A. niger*
L1	10	10	11	9	6	11
L2	9	12	10	8	6	6
Ceftriaxone	40.0	50.0	38.0	44.0		
Amphotericin-B					17.0	8.0

- The molecular docking study is missing a control to properly analyses the results.

Response:

We appreciate the recommendation and updated the molecular docking study by adding more details and literature references as follows,

4.9. *Molecular Docking Study*

Molecular docking is a powerful tool to investigate and provide a proper understanding for ligand receptor interactions in order to facilitate the design of potential drugs [93-96]. To investigate and compare the antimicrobial activity of the synthesized compounds, docking analysis of L1 and L2 against tyrosinase from *Bacillus Megaterium* were performed. It is well-known that tyrosinase of *Bacillus Megaterium* bacteria is an attractive target for the development of antimicrobials or antibiotic adjuvants for the treatment of hyperpigmentation because of its similarity (33.5%) to the human enzyme [97-100].

- Conclusion: Repeated abstract. The conclusion must remind the reader why the article was written in the first place and why it is important in the field. The conclusion should briefly give an insight into the obtained results and also the limitations.

Response:

We appreciate the recommendation and changed the conclusion as follows,

Pyrrole and thiophene as organic molecules and their metal cluster derivatives have been recognized to present a wide range of biological activities in recent years. In this present study we have synthesized two tritopic dihydrazide based ligands bearing Pyrrole and Thiophene as end groupings and characterized successfully by FT-IR, ^{1}H and ^{13}C NMR and mass spectrometry. Based on the DFT the calculations, a complete structural detail, vibrational, electrostatic potential, Mulliken population, HOMO-LUMO and thermodynamic analysis were also done. The computed FT-IR analysis as well as the ^{1}H and ^{13}C NMR using B3LYP/CC-PVDZ/6-311+G(d, p) method agreed satisfactorily with the experimental results. We further evaluated the thermodynamic parameters $\Delta \boldsymbol{H}$, $\Delta \boldsymbol{S}$, and $\Delta \boldsymbol{G}$ of the ligands. The geometry optimization revealed the planarity of L1 and L2 molecules. Further, it was seen from the HOMO-LUMO energy values that the chemical potentials were negative and the frontier orbital gap of the molecule under investigation was small, and hence, both compounds are reactive and polarizable. To further showcase the biological activity of the ligands against organic pathogens, the antimicrobial assay was performed and revealed significant inhibition of L2 against *Bacillus Megaterium* gram positive bacteria, and L1 against *Escherichia coli*, *Aspergillus niger* although in lesser extent. The moderate activity of L2 molecule against *Bacillus Megaterium* is substantiated by molecular docking study against tyrosinase from *Bacillus megaterium* and was found significant with a binding energy of -8.8 kcal/mol and three hydrogen bond interactions, which might suggest the antimicrobial activity of the molecule. Overall, L1 and L2 compounds have spurred significant interest for us from the synthetic, computational and biological point of view. We anticipate continued research regarding these classes of exciting organic ligands.

We also revise the whole manuscript to make the language and grammar better as suggested by the reviewer.

Dr. Md Abdul Majed Patwary

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