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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 #1:

1. Comment:

The manuscript by Sharmin Akther Rupa et al, the manuscript has many serious concerns on the basis that it cannot be accepted in this journal. for example, ^{13}C NMR must be 100 MHz not 400 MHz, value must be either ascending order or descending order (^{13}C NMR (400 MHz, DMSO-d6, ppm): 14.82 (-CH_3), 149.25 (-C=N-), 153.65 (Ar-C), 125.26 (Ar-C), 140.31 (Ar-C),159.11 (C=O), 130.04 (Ar-C), 112.83 (Ar-C), 109.34 (Ar-C), 123.05 (Ar-C). above example showed the manuscript not written carefully.

Response:

We appreciate the recommendation and comments. We corrected the above-mentioned correction as suggested by the reviewer and presented as follows,

^{1}H NMR (400 MHz, DMSO-d_6, ppm): δ 11.43 (s, 2H), 8.34 (m, 2H), 8.28 (m, 1H), 7.65 (d, 2H, J = 4.8 Hz), 7.61 (d, 2H, J = 3.2 Hz), 7.14 (t, 2H, J = 8.8 Hz), 2.51 (s, 6H). ^{13}C NMR (100 MHz, DMSO-d_6, ppm): 159.44 (C=O), 154.55 (Ar-C), 148.91 (Ar-C), 143.22 (-C=N-), 140.48 (Ar-C), 130.06 (Ar-C), 129.44 (Ar-C), 128.20 (Ar-C), 125.75 (Ar- C), 15.32 (-CH_3).

2. Comment:

Second one that is very important for this paper: The activity of L2 molecule against Bacillus Megaterium (PDB 4j6u) was significant with a binding energy of -8.8 kcal/mol and three hydrogen bond interactions, which reveals the antimicrobial activity of the molecule. Both compounds are biologically active, but their activity was moderate which did not support their

efforts.

Response:

We appreciate the recommendation and agree with the reviewer that the computational and experimental results of antimicrobial activity differ to some extent in this part of study and required further investigation to understand why such activity differ to the ligands by other means in future. In this study, we successfully synthesized the novel ligands and studied its spectroscopy with other probable studies. Fortunately, we have found outstanding results during chemo-sensor study and hopefully we could be able to show the excellent usage of the ligands as a chemo-sensor in near future. Also, we are planning to do fluorescence imaging study of protein-ligands.

3. Comment:

Third one their binding study: 1. Why did the authors consider Tyrosinase from Bacillus megaterium N205A mutant pdb id 4j6u? As they did not report any enzyme specific inhibition related experiments. 2. Docking is a preliminary experiment. How did the author validate the docking protocol? The authors are suggested to use some decoy ligands and calculate the enrichment value in order to justify the docking protocol followed by ALA scan (Alanine scanning) based MD experiments. In addition, authors are suggested to report at list 100ns Molecular dynamics (MD) simulation guided protein-ligand stability report followed by either MM-GB or PB-SA based thermo data analysis. 3. The pdb id 4j6u does not contain any bound ligand. How did the author select the binding site? If a few sets of amino acids were considered to define the binding site, then on what basis they selected them? 4. The authors did not report any Tyrosinase enzyme inhibition assay, they are requested to justify Tyrosinase inhibition as the probable mechanism of action for these sets of ligands by providing suitable literature references.

Response:

We appreciate the comments of the reviewer and following are the answers in response to the question for the kind consideration of the reviewer-

Tyrosinase is a key enzyme in melanogenesis, which is essential for pigmentation. Dysfunction of tyrosinase may cause skin cancer. 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 [97-103]. That's why, to investigate and compare the antimicrobial activity of the synthesized compounds with *in vitro* data, docking analysis of L1 and L2 against tyrosinase from *Bacillus Megaterium* were performed. Also, molecular docking with other proteins of *B. Megaterium* were conducted, but 46ju provided better binding affinity with the synthesized compounds. For this reason, we aim to study structure of tyrosinase from the bacteria.

Preliminary antimicrobial studies were carried out to validate the docking protocol. At present, we are doing the fluorescence activities of these ligands and L2 showed excellent fluorescent emission at λ_{max} 520 nm. So, we hope that we will carry out ALA scan (Alanine scanning) based MD experiments and 100ns Molecular dynamics (MD) simulation guided protein-ligand stability in future for our next study, since within this shortest time, we are unable to do these experiments.

The active binding site of tyrosinase was predicted by CASTp. The binding site residues predicted by CASTp for tyrosinase were used for grid generation. We have included this in the manuscript 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].

4.9.1. *Binding affinity of L1 and L2*

The highest anti-bacterial activity (zone of inhibition 12 mm) of compound L2 was detected with tyrosinase from *Bacillus Megaterium* (PDB ID: 4j6u) bacteria compared to L1. The binding energies for L1 and L2 with *Bacillus Megaterium* were −7.7 and −8.8 kcal mol^{-1}, respectively, which were calculated by AutoDock Vina. The interactions of the 4j6u with compounds L1 and L2 are shown in the Fig. **6**.

In L1-4j6u, one conventional hydrogen bond (3.04 Å) of O-H----O-C observed between O-H of Tyr267A and O-C group of compounds L1. Pi-cation, pi-sulfur and amide-pi bonds were

also noticed with LYS47A, PHE48A, ILE39A, ALA40A, GLY43B and ALA44B, respectively. Moreover, ALA44A, LYS47A, ALA44B, LYS47B, PRO52A, ALA40B and ILE139B were actively involved in the non-covalent interaction (hydrophobic pi-alkyl). L2-4j6u complex was stabilized by four NH….O hydrogen bonds and they were LYS47A (2.25 Å), GLY143B (3.04 Å), Tyr267A (3.07 Å) and PRO219B (2.91 Å) (Fig. **6**). Like L1, L2 formed pi-cation and amide-pi bonds with LYS47A, ILE39A, GLY43B, where the distances were 3.73, 4.34, 3.54 Å. L2 also formed seven pi-alkyl bonds with ALA44A (5.19 Å), LYS47A (4.35 Å), ALA44B (3.95 Å), LYS47B (4.98 Å), PRO52A (5.04 Å), ALA40B (4.67 Å) and ILE139B (4.24 Å), respectively. Results of docking studies revealed that L1 and L2 formed bonds to the active site of tyrosinase and showed strong interactions with Tyr267A, Ala44A, and ALA44B of tyrosinase enzyme (PDB ID: 4j6u), which also supports the literature [101-103].

Thus, computational results are in good agreement with *in vitro* antibacterial behaviour of our compounds for novel antibacterial drug design.

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

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