Original Article

Discovery of Potential Inhibitors of *Mycobacterium tuberculosis* EthR Using Structure and Ligand Based *in silico* Approaches

Bandar Hamad Aloufi*, Ahmed Mohajja Alshammari

Department of Biology, College of Science, University of Ha'il, Ha'il, SAUDI ARABIA.

ABSTRACT

Aim and Background: *Mycobacterium tuberculosis* (TB) remains the leading cause of human death posing one of the most serious threats to public health around the world. New strategies need to be developed to combat the growing danger by multidrug resistance. The present study aims to screen three different compounds inhibiting the binding pocket of Regulatory Repressor Protein EthR of *Mycobacterium tuberculosis*. In this study we performed pharmacophore modeling based virtual screening to identify the potential inhibitors against EthR of *Mycobacterium tuberculosis*. Based on the binding energy and hydrogen bond interactions three compounds were selected as potential inhibitors. **Materials and Methods:** Structure of EthR protein (PDB ID: 5NZ0) was retrieved from pdb databank. Further, we retrieved ligands from ZINC database (ZINC223412753, ZINC030691754, ZINC170602403). Next, Computational screening, Docking studies and Molecular dynamic simulations were performed to validate the stability of the complexes. **Results:** The molecular docking showed that all ligands interact with EthR protein of Mycobacteriam. Further, molecular dynamics simulation showed that ligand ZINC223412753 form comparatively more stable complex with EthR. Results showed that all the three ligands could be a potential inhibitor of EthR. **Conclusion:** These compounds can serve as a starting point in rational design of selective potent inhibitors against *Mycobacterium tuberculosis*.

Keywords: *Mycobacterium tuberculosis*, Pharmacophore, Molecular Docking, Virtual Screening, Molecular Dynamic Simulations, Inhibitors.

INTRODUCTION

Tuberculosis (TB) is the most common cause of human death from a treatable infectious disease. The disease is caused by aerosol transmission of *Mycobacterium tuberculosis*. According to the WHO (World Health Organization) approximately 2 million deaths and 9 million new infections worldwide each year was reported. Total incidence of tuberculosis (TB) rises about 0.3 percent every year, mainly in resource-poor countries, including areas with high levels of poverty and lack of infrastructure and medical facilities.[1-2] The growth of multidrug-resistant tuberculosis (MDR-TB) strains are the main cause of resurrection of TB.[3] Despite the fact that effective chemotherapies exist, no new medications have been introduced to the market in the last 40 years. Furthermore, rising drug resistance among *M. tuberculosis* strains is posing a serious threat to public health, as evidenced by the emergence of highly drug-resistant tuberculosis recently (XDR-TB),which has resulted in a fatality rate of >98%.[4] Thus, there is a need to discover and develop new and better tuberculosis treatment drugs, especially drugs that reduce treatment duration and treat patients with multi drug resistant strains. Furthermore, the global HIV epidemic has aided in the rapid spread of tuberculosis (TB) and the rise of multi-drug-resistant (MDR) strains. The number of people infected with MDR-TB was likely to be 650,000 in 2010.[5] A tuberculosis patient must be treated with multiple second-line drugs for 2 to 4 years.

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Correspondence:
Dr. Bandar Hamad Aloufi
Department of Biology,
College of Science,
University of Ha'il,
Ha'il-2440,
SAUDI ARABIA.
E-mail: bandaraloufi@yahoo.com

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These drugs have poor efficacy and poor tolerability, weakening observance and leading to treatment failure.[6] Present tuberculosis treatment includes a vast majority of prod rugs that must be metabolically activated before they can work.,[7] whereas pyrazinamidase PncA activates pyrazinamide pro-drug,[8] and ethionamide, isoxyl and thiacetazone is activated by monooxygenase EthA.[9-11] In recombinant mycobacteria, overproduction of the prod rug activators KatG and EthA has been found to significantly improve sensitivity to the corresponding prod rugs.[12-13] As a result, the antimycobacterial efficacy of these prodrugs appears to be restricted *in vitro*, by the rate of bio activation. This means that only a small amount of prod rugs used to treat tuberculosis are effective against the bacteria. Ethionamide is a second-line tuberculosis drug that is used to treat individuals with multidrug-resistant *Mycobacterium tuberculosis* infections. This prod rug is activated by flavin-dependent monooxygenase enzyme EthA. The transcriptional repressor EthR controls the expression of the mycobacterial EthA. Intracellular levels of EthA determine the toxicity issued caused by the large effective dose of ethionamide in patients.[14]

Small compounds that bind to EthR have been demonstrated to allosterically block the EthR dimer's DNA-binding capacity, effectively removing its role as an EthA transcriptional repressor. Many studies have shown that the compounds which bind to EthR can serve as ethionamide enhancer in the detection of whole-cell *Mycobacterium tuberculosis*.[15-17] The ineffectiveness of ethionamide activation necessitates the use of high therapeutic doses, which are frequently accompanied by severe side effects, restricting its usage as a second-line medication against drug-resistant strains.[18-19] This study aims to screen three different compounds targeting the binding pocket of EthR for inhibiting the DNA-binding function of EthR. Pharmacophore model was developed to search against the large data set of the compounds. Further Molecular dynamics simulations and binding energy analysis were performed to validate the binding of the potential binders against the Transcriptional Regulatory Repressor Protein EthR of *Mycobacterium tuberculosis*.

MATERIALS AND METHODS

Virtual screening techniques are widely used in the design and development of novel drugs. Structure-based virtual screening (SBVS) is one of the most used virtual screening techniques, as it just requires the three-dimensional structure of the target protein. It has been proven that structure based drug designing is effective as compare to traditional drug designing methods because it aims to comprehend the molecular foundation of a disease, by utilizing information of the structure of the target.[20] SBVS have been used to discover several drugs.[21-25]

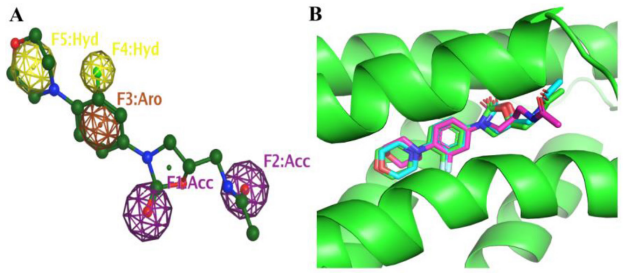


Figure 1: A) Pharmacophore Model features used to search against the three datasets of compounds B) Redocked conformations (bound docking as blue color and unbound docking in magenta color) of linezolid inhibitor superimposed to native conformation (green).

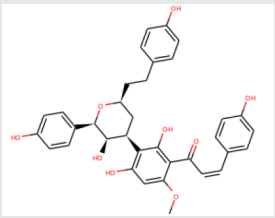
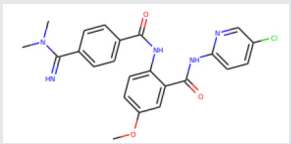
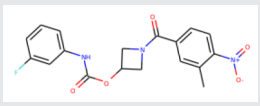
Receptor Preparation

The crystallographic structure of *Mycobacterium tuberculosis* EthR was downloaded from the Protein Data bank (PDB ID: 5NZ0). All the water molecules and other crystallographic molecules were removed from the structure before the docking. AutoDock Tools,[26] was used to prepare the receptor for docking study. Only polar hydrogens were added to the protein structure. Gassteiger charges were also computed of the protein structure before docking. Later on the receptor structure was saved in PDBQT formate for docking.

Pharmacophore modeling and Tanimoto coefficient similarity

There different datasets of the ligands were used in this study, MPD3 medicinal plant database (2296 compounds) and FDA-approved ligand dataset (1616 compounds), and Drug like molecules (436377 compounds) were downloaded from the ZINC15 database.[27-28] Similarity search was conducted against the target databases using the tanimoto coefficient (TC). Linezolid inhibitor was used as a reference to search the similar compounds in the databases. Openbabel was used for Tanimoto coefficient similarity searching.[29] To augment the dataset of the compound for docking a pharmacophore based screening was also conducted sing linezolid complexed with *Mycobacterium tuberculosis* EthR. The MOE was used to build the pharmacophore model and pharmacophore search against the target databases.[30] Pharmacophore based screening was done using following feature; one aromatic feature, two hydrogen bond acceptor features, and two hydrophobic (Figure 1). After the similarity and

Table 1: Chemical attributes of the final selected compounds.

Compound ID	Chemical Structure	logP	Molecular Weight	H-bond Donor	H-bond Acceptors
ZINC223412753		5.7	598.64	6	9
ZINC030691754		4.1	451.91	3	4
ZINC170602403		3.1	373.34	1	5

pharmacophore search all the ligands were prepared using AutoDock Tools for docking. Polar hydrogens and gassteiger charges were added to the ligands. After preparing all the ligands the ligand files then saved in PDBQT format for docking purpose.

Molecular Docking

The filtered compounds based on TC similarity and pharmacophore searching were then docked into the active site of the *Mycobacterium tuberculosis* EthR using AutoDock Vina.[31] The binding pocket of *Mycobacterium tuberculosis* EthR was defined on the bases of binding of the linezolid inhibitor in the active site of *Mycobacterium tuberculosis* EthR (PDB ID: 5NZ0). The protein was kept in rigid state during docking. Size of the grid box was set (50x50x50) and grid box was placed at x = 3.107, y = 24.488, z = -11.980. All the docked ligands were then ranked and filtered based on the binding energy (Kcal/mol) and hydrogen bond interactions.

Molecular Dynamic Simulations

Molecular dynamics (MD) simulations have widely been used to study biomolecules. MD simulation was used to investigate the conformational changes, stability of the protein-ligand complexes using GROMACS (GROningen Machine for Chemical Simulations).[32] The charmm36[33] forcefield was used to prepare the topologies of each of the systems and the complexes were placed in a cubic box and solvated with the SPC216 water model based on periodic boundary conditions. The net charge of the systems was neutralized and each of the systems was minimized by the steepest descent algorithm at 50000 steps until a convergence tolerance of 1000KJmol-1min-1 was reached. Then each of the systems was equilibrated with NVT for 1ns at 310K and NPT at 1bar. LINCS algorithm was used to restrict all bonds.[34] Finally, the equilibrated systems were subjected to 100ns MD simulation, and the trajectories generated were used to analyze the stability of the ligand-protein complexes.

MMGB/PBSA Analysis

Molecular mechanics/Poisson–Boltzmann surface area (MM/PBSA) method was used to calculate the binding energy (ΔG) of the complexes. This method has been widely used to calculate the binding free energy of docked complexes.[35-37] In order to have full details of the protein-ligand complexes interactions, the binding energies of each of the protein-ligand complexes were calculated using the MM/PBSA protocols.[38]

RESULTS AND DISCUSSION

Validation of the Docking Protocol

Molecular docking was performed to identify potential inhibitors against *Mycobacterium tuberculosis* EthR. To assess the predictive ability of the docking protocol used in this study bound and unbound docking of the linezolid inhibitor was performed. For bound docking, the experimentally determined crystallographic conformation of the ligand linezolid was detached from the *Mycobacterium tuberculosis* EthR structure and then re-docked and for unbound docking, a different conformation of the linezolid inhibitor was downloaded from the PubChem database, and docked into the active site of *Mycobacterium tuberculosis* EthR. RMSD

Table 2: Binding Energy and Hydrogen bond interactions of the final selected compounds.				
Compound ID	**Compound Name**	**Energy Kcal/mol**	**Interacting Residues**	**Source**
ZINC223412753	6-hydroxycalyxin F	-10.6	Leu90, Asn93, Tyr148, Thr149, Arg159	Natural mpd3
ZINC030691754	Betrixaban	-9.7	Tyr148, Thr149	FDA
ZINC170602403	1-(3-methyl-4-nitrobenzoyl) azetidin-3-ylN-(3-fluorophenyl) carbamate	-9.4	Tyr148, Thr149	Druglike

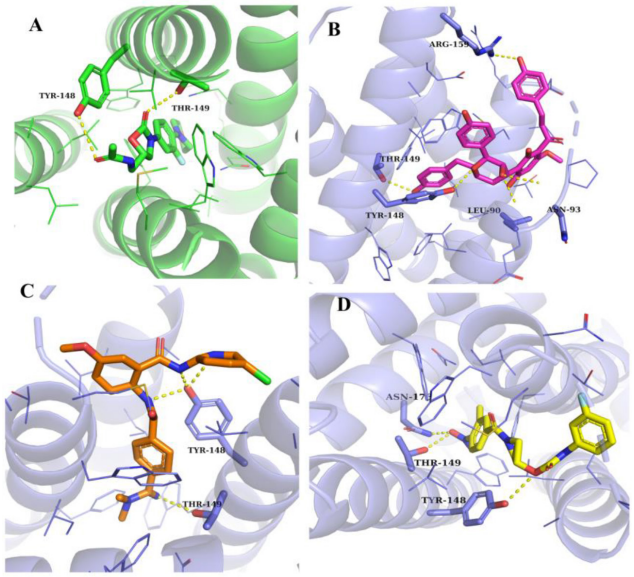


Figure 2: Binding conformations of the final selected compounds in the pocket of *Mycobacterium tuberculosis* EthR, Residues making hydrogen bonds with the compounds are shown as sticks and labeled.

A) Crystallographic ligand B) ZINC223412753 C) ZINC030691754 D) 1 ZINC170602403.

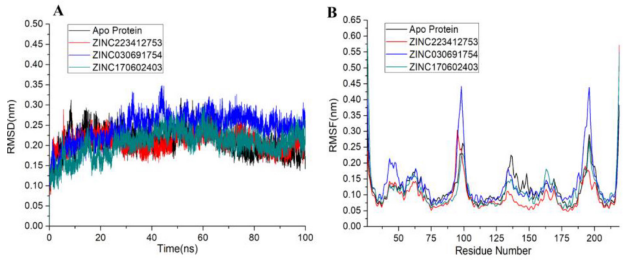


Figure 3: (A) Root mean square deviations (RMSD) (B) Root mean square fluctuations and of the complexes and apo protein over the time of 100ns.

of bound docking and unbound docking concerning the co-crystallized ligand was 0.37Å and 1.39Å dash with binding energy -9.1kcal/mol and -9.7 kcal/mol respectively (Table 1). Figure 1 shows the superimposed conformations of the re-docked ligands on the co-crystallized ligand. RMSD of re-docked ligands demonstrated that the docking procedure is robust enough to achieve the physically related binding pose. Figure 1 shows the superimposed conformations of the re-docked ligands on the co-crystallized ligand.

Molecular Docking

The top 90 compounds were selected and visually inspected based on binding energy score of the crystallographic ligand. The ligand-protein interactions were analysed, compounds making hydrogen bonds with the important pocket residues were selected as potential inhibitors. The results in Table 2 shows that compound ZINC223412753 showed the highest binding score -10(kcal/mol). Compound ZINC223412753 is a natural compound belonging to flavonoids. This compound made five hydrogen bond interactions with residues Leu90, Asn93, Tyr148, Thr149, Arg159. Compound ZINC030691754 was ranked second with the binding energy -9.7(Kcal/mol) and ZINC170602403 was ranked at third with binding energy -9.4(Kcal/mol) forming hydrogen bonds with Tyr148, Thr149. Compound ZINC030691754 ranked second is also an FDA-approved drug for venous thrombosis prevention in adults.[39] All the compounds were making two common hydrogen bond interactions with resides Tyr148, Thr149. The co-crystallized ligand linezolid also shows two hydrogen bonds one with the side-chain OH group of Tyr148 and one with side-chain OH of Thr149 (Figure 2).

Molecular Dynamic Simulations

To further explore the dynamic behavior of all the protein-ligand complexes molecular dynamic (MD) simulations were performed. MD simulation is a widely used method to investigate dynamic behavior of the ligand-protein complexes. After the MD simulations RMSD, RMSF, Rg, ligand-protein interaction analysis and MMGBSA analysis were performed.

Root Mean Square Deviation

The stability and the conformational changes in the secondary structure features of the protein-ligand complexes were observed using the root-mean-square

Table 3: Binding energies (kJ/mol) analysis of the protein-ligand complexes.					
Compound ID	ΔE_{elec} (KJ/mol)	ΔE_{vdw} (KJ/mol)	ΔESA (KJ/mol)	ΔE_{polar} (KJ/mol)	$\Delta E_{binding}$ (KJ/mol)
ZINC223412753	-12.575±19.40	-79.834±106.87	-9.108±12.38	67.840±71.980	-33.677±80.807
ZINC170602403	-3.300±4.47	-50.766±87.047	-5.057±8.699	26.849±62.666	-32.273±56.848
ZINC030691754	-7.644±13.70	-61.555±89.621	-6.933±10.13	47.500±58.834	-28.632±63.410

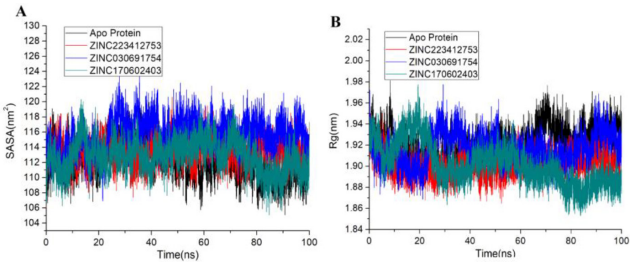


Figure 4: (A) solvent-accessible surface area (B) Radius of gyration of all the complexes and Apo protein.

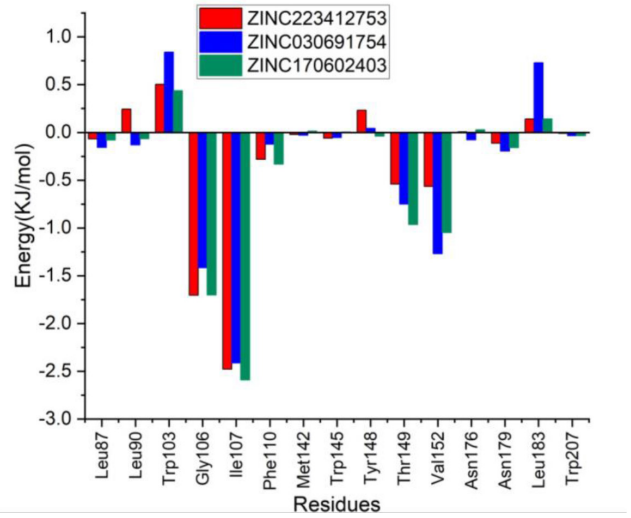


Figure 5: Energy decomposition analysis of the binding pocket residues of all the complexes.

deviations Figure 3. The RMSD of the backbone atoms of the protein-ligand complexes when compared with the Apo protein was used to infer the stability and the fluctuation of the protein-ligand complexes. The complexes reached equilibrium and remain constant throughout the simulation time implying that they reached a stable state while the ligands are bonded in the target protein binding sites.

Root Mean Square Fluctuation (RMSF)

The root mean square fluctuation allows for the estimation of the average fluctuation observed in the protein-ligand complexes. From our RMSF plots, we observed high peaks at the beginning and towards the end of the target protein Figure 3. The high peaks indicate the most fluctuating residues in the protein. The amino acid residues of all the complexes tend to have minimal fluctuation while the ligands are bonded in the binding sites. However, compounds ZINC030691754 and ZINC223412753, have some fluctuations around residues 90 – 110 and compound ZINC170602403 has a slightly lower fluctuation between these residues. A high peak was also observed around residues 170-210 in complex ZINC030691754. Overall, all the complexes have less fluctuation as compare to the Apo protein. This indicates that the protein-ligand complexes are relatively stable.

Solvent Accessible Surface Area (SASA) and Radius of Gyration (Rg)

All the complexes and the apo protein were used to calculate the solvent-accessible surface area, SASA Figure 4. SASA is also a major contributor to understand the ligand-protein binding. The extent of the ligand binding to the receptor amino acid residues is such that they have accessibility to solvents. The SASA plot describes that the amino acid residues of complex ZINC170602403 tend to have similar solvent accessibility to the apoprotein. The compound ZINC030691754 and ZINC223412753 residues also have exposure to the solvent within the environment. To check the compactness of the system, radius of gyration (Rg) was also analysed, which has an impact on the folding rate of protein Figure 4. The compound ZINC030691754 and ZINC170602403 complex Rg was a bit higher than the apoprotein, there was an increase slightly at 70ns and compound ZINC223412753 maintain a regular pattern from 70ns till the end of the simulations. The further shows the protein-ligand complexes are relatively stable.

MM/PBSA Binding Free Energy Calculation

The binding energies of the ligands to the target protein were determined using the Molecular Mechanics-Poisson Boltzmann Surface Area (MMPBSA). The 100-ns simulated trajectories were used for the analysis. The bind energy of complex ZINC223412753 is -33.677±80.807 kJ/mol, ZINC030691754 is -28.632±63.410 kJ/mol and for complex ZINC170602403 is -32.273±56.848 kJ/mol. In complexes ZINC223412753 and ZINC030691754

the ΔEvdw, ΔEelec and ΔESA energy significantly contributed to the binding energy, while in case of complex ZINC17060240 ΔEelec was quite large as mentioned in the Table 3. To further gain insights into the ligand-protein energy contribution of each residue was calculated Figure 5. Gly106, Ile107, Phe110, Thr149, Val152 were the major contributors in ligand-protein binding. Besides this Leu87, Leu90, Tyr148, Asn179 also favored the binding of ligands with *Mycobacterium tuberculosis* EthR.

CONCLUSION

The process of drug development and drug discovery is a significant process that is money and time-consuming. In this process, computational techniques are commonly used by pharmaceutical industries as an initial step to save time and money. In this study, we present a computational approach as initial step to identify compounds that act as inhibitors against *Mycobacterium tuberculosis* EthR, which requires *in vitro* studies to assess the efficacy of the compound. the molecular docking study revealed 6-hydroxycalyxin F, Betrixaban, and 1-(3-methyl-4-nitrobenzoyl) azetidin-3-yl N-(3-fluorophenyl) carbonate have shown significant binding affinity with active site of the target protein. All three compounds were making hydrogen bonds with the important amino acids in the active site (Tyr148, Thr149). Further MD studies also revealed the strong binding affinity and stability of the complexes indicating that these compounds may serve as potential inhibitors against Transcriptional Regulatory Repressor Protein EthR of *Mycobacterium tuberculosis*.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest.

ABBREVIATIONS

EthR: Ethionamide Receptor; **ZINC223412753:** 6-hydroxycalyxin F; **ZINC030691754:** Betrixaban; **ZINC170602403:** 1-(3-methyl-4-nitrobenzoyl)azetidin-3-yl N-(3-fluorophenyl)carbamate; **MPD3:** Medicinal Plant Database for Drug Designing; **FDA:** Food and Drug Administration.

Author contributions

Bandar Hamad Aloufi and Ahmed Alshammari have planned and designed the experiment, collected and analyzed the data and have written the article.

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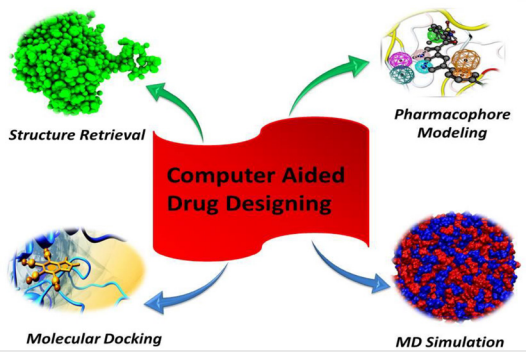
PICTORIAL ABSTRACT



SUMMARY

Mycobacterium tuberculosis is the leading cause of human mortality. It is globally considered as the most serious hazard to public health because of its property of multidrug resistance. In order to combat this multidrug resistance, novel strategies must be developed. In such instance, the Ethionamide Resistant (EthR), Regulatory repressor protein of *Mycobacterium tuberculosis* might be an appealing target. In the current study, pharmacophore based virtual screening was performed to identify the potential inhibitors against the EthR of *Mycobacterium tuberculosis*. Three compounds were selected as potential inhibitors based on their binding energy and hydrogen bond interactions. To further validate the stability of these interactions, molecular dynamic simulations were performed. It was observed that these compounds are in fact inhibitors of EthR. In conclusion, these compounds have the potential to counter the threat of Multidrug resistance by serving as the initial point in designing a rational approach towards selective potent inhibitors against *Mycobacterium tuberculosis*.

About Authors

Bandar Hamad Aloufi: Aloufi is an expert in molecular Biology and biotechnology, and his research interests in systematic biology and bioinformatics relate to biological and physiological studies. Dr.Aloufi has authored many articles in reputed journals. He has also published many textbooks in biology and physiology. He has participated in various national and international conferences and isa member of many national and international academic associations.

Ahmed Mohajja Alshmmari: Alshammar is an associate professor of biology and has a Ph.D. in zoology in the field of Ecological sciences. His research interests in systematic biology and bioinformatics relate to biological, toxicological, and physiological studies. He is the author of six books and forty manuscripts (including published and submitted). He has published many books in biology and physiology. He has participated in various national and international conferences and is a member of many national and international academic associations.

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