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NEW INHIBITORS AGAINST *accD3* GENE OF *MYCOBACTERIUM TUBERCULOSIS, IN-SILICO* STUDY AND *IN -VITRO* APPLICATION

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ABSTRACT: *Mycobacterium tuberculosis* (Mtb) is a pathogenic bacteria species in the genus Mycobacterium and the causative agent of most cases of tuberculosis. Tuberculosis (TB) is the leading cause of death in the world from a bacterial infectious disease. This antibiotic resistance strain lead to development of the new antibiotics or drug molecules which can kill or suppress the growth of *Mycobacterium tuberculosis*. The aim of this study is discovery a new drug molecules against the product of *accD3* gene which play a vital role in mycolic acid biosynthesis pathway (MAP). As a first step *accD3* gene amplified from the complete genome of *Mycobacterium tuberculosis* Iraqi isolates and sequenced, eight out of twenty of the resulted sequences were chosen to achieve the *in silico* studies, these sequences have deposited in GenBank database with strain names (ALQM1, ALQM2, ALQM3, ALQM4, ALQM5, ALQM6, ALQM7, ALQM8) and Accession numbers (LC006979, LC008196, LC009412, LC009414, LC034168, LC038020, LC041163, LC041368) respectively, two drug molecules resulted from *in silico* studies and used in the practical application to prove the *in vitro* inhibitory ability of these molecules on viable cells of *M. tuberculosis*, REMA which is a colorimetric assay was used in this research to estimate the inhibitory application of these molecules.

INTRODUCTION: Tuberculosis (TB) proved to be a controllable, preventable and curable disease, but it still remains as a leading cause for mortality[1]. It was declared a global health emergency in 1993 by the World Health Organization (WHO) when approximately 8 million Tb cases were estimated and 1.3-1.6 million deaths were occurred from the disease each year, now TB exacerbated by the spread of AIDS.

QUICK RESPONSE CODE

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Iraq has a high burden of TB and ranks 44th worldwide among countries with a high TB burden and 7th among the countries of the Eastern Mediterranean region. Anti - tuberculosis (TB) drug resistance is a major public health problem that threatens progress made in TB care and control worldwide. Rational Drug Design (RDD) is basically a computer-aided molecular modeling, it is an iterative process, If it is based on the knowledge of three - dimensional (3-D) structure of the target proteins of interest; it is called "structure based drug design" or "target based drug design". Eight nucleotide sequences were extracted from Iraqi Mtb isolates were used in this study, two molecules gave a good results in the inhibition of *M. tuberculosis* growth by using REMA test which is a colorimetric assay [2].

The main aim of this study is the discovery of new drugs against Tuberculosis.

MATERIALS AND METHODS: Sputum samples were collected from 200 Iraqi suspected patients who attended the Institute of Chest and Respiratory diseases / Baghdad, for the period from 1st of May 2013 to 31st July 2013. Their ages were ranging from 8 years up to 85 years, 50 of the total 86 positive cultures were randomly chosen to complete this study.

DNA Extraction from *M. tuberculosis* isolates and primer design: DNA was extracted from pre diagnosed mycobacterial cultures and purified by CTAB method [3, 4]. *M. tuberculosis* isolates were characterized at molecular level using *LprM* gene, primer pairs were used to amplify this gene to differentiate *M. tuberculosis* from the other types of mycobacteria [5, 6]. The results showed that 43 isolates (86%) among of 50 isolate were *M. tuberculosis*.

DNA sequences of *accD3*(1488 bp) was retrieved from puplic database, primers were designed for the gene at two position, segment I from (38-831) to give PCR product 793 bp. Segment II from (631-1482) to give PCR product 851 bp. There was an overlapped sequence about 300 bp. Which were curated manually. The resulted sequence covered the region from(39-1482), these were aligned using Clustal W, and phylogenetic tree was built using NJ method, all these sequences were deposited at NCBI/nucleotide (GenBank database) with Accession numbers (LC006979, LC008196, LC009412, LC009414, LC034168, LC038020, LC041163, LC041368). The primers sequences used in PCR experiments are shown in **Table 1.**

TABLE 1: PRIMER PAIRS FOR *accD3* GENE SEGMENTS

Segment I (SI) (38-831)	Forward	GCTAGACCGGGGATCTTTCG	PCR product	793bp
	Reverse	GCCTTGATCGGTTCCTGACA		
Segment II (SII) (631-1482)	Forward	TGAGTTGCTCTATGGCGACC	PCR product	851bp
	Reverse	GACAGTCGTAGGGCGAACTC		

Amplification of Target gene: *AccD3* gene segments (SI and SII) were amplified separately. Each PCR mixture was prepared with 25 µl of Green Master Mix 2x(Promega), 17µl of nuclease free water, 2µl of each primer (F,R) at 10µM, and 4µl of DNA (equaling 25 to 250 ng). Multiple PCR programs were used until reach to the optimum program which gives a good PCR product, the thermocycling conditions for SI and SII were 94 °C for 5 minutes, followed by 35 cycles of denaturation at 94 °C for 1 minute , and annealing at 59 °C for 1.30 minute and 72 °C for 1 minute, then the final extension steps at 72 °C for 10 minutes. PCR amplified products (10µl) and DNA molecular –weight marker (Ladder) were electrophorized on 1% Agarose gel with ethidium bromide staining to verify the size of the amplicon, the resulted PCR product were sequenced (NICEM-USA, Apparatus: Applied Biosystem).

Computer aided drug design and Discovery steps:

Target Determination: The following programs and databases were used for target determination:

A. NCBI Database [7]
(http://www.ncbi.nlm.nih.gov/)

B. Uniprot Database [8]
(http://www.uniprot.org/)

C. KEGG Database [9]
(http://www.genome.jp/kegg/)

D. TMHMM server v.2.0 **[10]**
(http://www.cbs.dtu.dk/services/TMHMM/

E. Tuberculist Database **[11]**
(http://tuberculist.epfl.ch/)

F. Mega6 software v 6.1 [12]

Homology Modelling: The following servers were used for protein modeling:

A. Swiss-Model Server [13, 14]
(http://swissmodel.expasy.org/**)**

B. QMEAN server:
(http://swissmodel.expasy.org/qmean/cgi/index.cgi**)** for the best protein model estimation. [15]

Ligand search and Pharmacophore building:

A. Zinc database [16]
(http://zinc.docking.org/)

B. Zinc Pharmer [17]
(http://zincpharmer.csb.pitt.edu/).

C. Discovery Studio software v.2.5: [18]

Toxicity and Mutagenicity Estimation:
A. Lazar software
(http://lazar.in-silico.ch/predict**)** [19]

B. T.E.S.T software v.4.1 [20]

***In vitro* application:**
Resazurin Microtiter Assay (REMA) is a Colorimetric Assay was used in this study to test the activity of drugs on the viable cells of *M. tuberculosis.* [21, 22]

RESULTS AND DISCUSSION: *M. Tuberculosis* contain multiple versions of *accD* genes that encode α and β subunits of at least three distinct multifunctional acyl CoA Carboxylase Complexes [23, 24]. The function of a number of genes involved in fatty acid and mycolic acid biosynthesis are known for their role in the survival of pathogenic *M. tuberculosis* [25, 26]. In this study, (*Rv1970*) gene was used to differentiate *M. tuberculosis* from the other of *M. tuberculosis* subspecies, *LprM* (*Rv1970*) is a gene lies in the RD7 region [27, 28]. In prior studies, PCR analysis revealed RD7 to be present in all of the tested *M. tuberculosis* but not in the evaluated *M. microti*, *M. bovis*, *M. bovis* BCG, and *M. caprae* strains [29, 30]. Primers were used to amplify *Rv1970* (*mce3E*) and the PCR results were positive for 40(80%) of isolates (Figure not shown).

Two PCR products of typical *M. tuberculosis* phenotype namely Asraa 1 and Asraa 2 were sequenced and deposited in NCBI with Accession numbers KJ809110, KJ809111 respectively and at DDBJ database with Accession numbers LC009880, LC009881.

In this study, *accD3* (*Rv0904*c) was used as a target protein for Computer Aided Drug Design because the product of this gene is acetyl-CoA carboxylase which catalyzes the first step in the pathway of fatty acid biosynthesis in *M. tuberculosis*. Acetyl - CoA is coupled with (Carbon mono Oxide) CO to form malonyl - CoA, which is used solely for the production of fatty acids and related compounds, this step is the main point for regulation of the entire pathway, thus making it an attractive target for the development of new drugs [31, 32].

H37Rv strain was used as the reference strain in this study, *accD3* (*Rv0904*) sequence of H37Rv was retrieved from NCBI and aligned with other *M. tuberculosis* strains to find the conserved regions in the *accD3* sequence among the different strains. **Fig. 1** shows the alignment results using Clustal W incorporated in Bio Edit program.

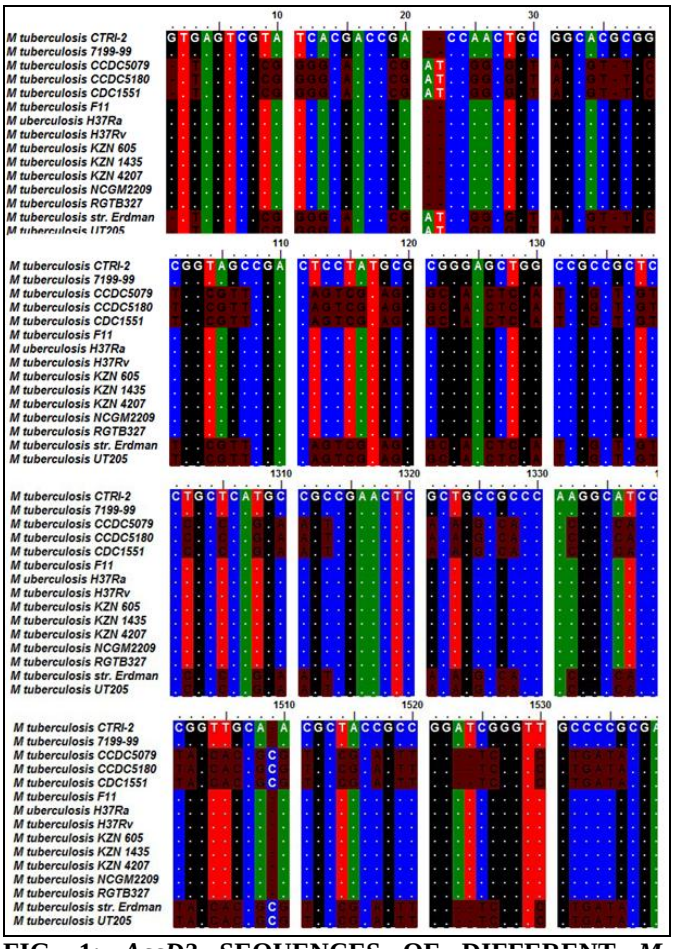


FIG. 1: *AccD3* SEQUENCES OF DIFFERENT *M. TUBERCULOSIS* STRAINS

The information about the target gene (*accD3*) were collected from VFDB (Virulence Factor of Pathogenic Bacteria) and Tuberculosis databases, *Rv0904*c (*accD3*) is a gene located at 1006693 – 1008180 from *M. tuberculosis* (H37Rv) genome and considered a relatively long gene (**Fig. 2**).

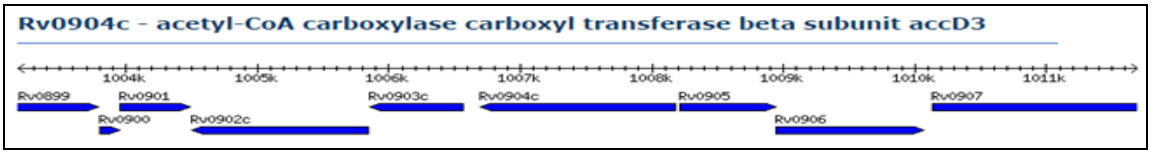


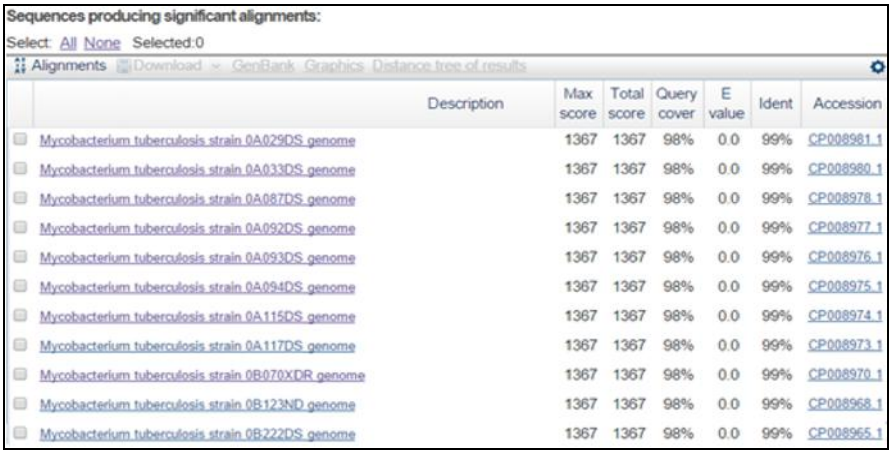
FIG. 2: GENOMIC LOCATION OF *Rv0904c*. TUBERCULIST DATABASE

Due to the long length of the gene, it was divided in to two segments (SI and SII). Primer pairs for SI able to amplify the region on genome at location (38-831) with PCR product 793 bp which represent the first half of target gene and primers pair for SII able to amplify the region (631-1482) with PCR product 851 bp which represent the second half of the target gene as shown in **Table 2**.

TABLE 2: PRIMER PAIRS FOR *accD3* GENE SEGMENTS DESIGNED WITH PRIMER-BLAST PROGRAM

Segment I (SI)	Forward Reverse	GCTAGACCGGGGATCTTTCG GCCTTGATCGGTTCCTGACA	PCR product	793bp
Segment II (SII)	Forward Reverse	TGAGTTGCTCTATGGCGACC	PCR product	851bp

The forty positive for *M. tuberculosis* isolates were subjected to PCR amplification and the designed primer pairs for the two segments of *accD3* gene were used to amplify the region of *accD3* gene at location 1006693-1008180 on the *M. tuberculosis* genome. (Figures not shown). The resulted products were sequenced. BLASTn at NCBI showed that these sequences were matched with *M. tuberculosis* at Query cover 98% for SI and 97% for SII and identity percentage reached to 99% at E-value 0.00 as shown in **Fig. 3** for SI and **Fig. 4** for SII.

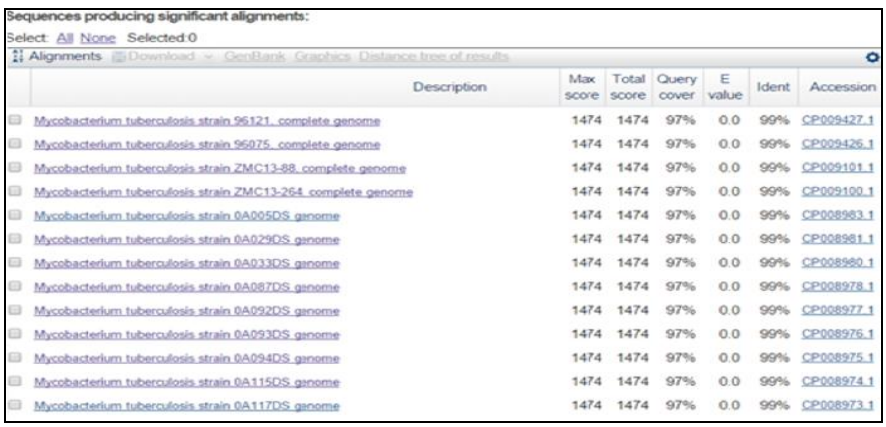

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycobacterium tuberculosis strain 0A029DS genome	1367	1367	98%	0.0	99%	CP008981.1
Mycobacterium tuberculosis strain 0A033DS genome	1367	1367	98%	0.0	99%	CP008980.1
Mycobacterium tuberculosis strain 0A087DS genome	1367	1367	98%	0.0	99%	CP008978.1
Mycobacterium tuberculosis strain 0A092DS genome	1367	1367	98%	0.0	99%	CP008977.1
Mycobacterium tuberculosis strain 0A093DS genome	1367	1367	98%	0.0	99%	CP008976.1
Mycobacterium tuberculosis strain 0A094DS genome	1367	1367	98%	0.0	99%	CP008975.1
Mycobacterium tuberculosis strain 0A115DS genome	1367	1367	98%	0.0	99%	CP008974.1
Mycobacterium tuberculosis strain 0A117DS genome	1367	1367	98%	0.0	99%	CP008973.1
Mycobacterium tuberculosis strain 0B070XDR genome	1367	1367	98%	0.0	99%	CP008970.1
Mycobacterium tuberculosis strain 0B123ND genome	1367	1367	98%	0.0	99%	CP008968.1
Mycobacterium tuberculosis strain 0B222DS genome	1367	1367	98%	0.0	99%	CP008965.1

FIG. 3: ALIGNMENT RESULTS OF *accD3* (SI)


Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Mycobacterium tuberculosis strain 96121, complete genome	1474	1474	97%	0.0	99%	CP009427.1
Mycobacterium tuberculosis strain 96075, complete genome	1474	1474	97%	0.0	99%	CP009426.1
Mycobacterium tuberculosis strain ZMC13-88, complete genome	1474	1474	97%	0.0	99%	CP009101.1
Mycobacterium tuberculosis strain ZMC13-264, complete genome	1474	1474	97%	0.0	99%	CP009100.1
Mycobacterium tuberculosis strain 0A005DS genome	1474	1474	97%	0.0	99%	CP008983.1
Mycobacterium tuberculosis strain 0A029DS genome	1474	1474	97%	0.0	99%	CP008981.1
Mycobacterium tuberculosis strain 0A033DS genome	1474	1474	97%	0.0	99%	CP008980.1
Mycobacterium tuberculosis strain 0A087DS genome	1474	1474	97%	0.0	99%	CP008978.1
Mycobacterium tuberculosis strain 0A092DS genome	1474	1474	97%	0.0	99%	CP008977.1
Mycobacterium tuberculosis strain 0A093DS genome	1474	1474	97%	0.0	99%	CP008976.1
Mycobacterium tuberculosis strain 0A094DS genome	1474	1474	97%	0.0	99%	CP008975.1
Mycobacterium tuberculosis strain 0A115DS genome	1474	1474	97%	0.0	99%	CP008974.1
Mycobacterium tuberculosis strain 0A117DS genome	1474	1474	97%	0.0	99%	CP008973.1

FIG. 4: ALIGNMENT RESULTS OF *accD3* (SII)

There was an overlap sequence with length about 300 bp between SI and SII, this was removed and merged using Mega 6 and merger tools at EMBOSSpackage(http://emboss.bioinformatics.nl/cgi-bin/emboss/merger) to obtain the whole sequence. The obtained sequences of *accD3* were BLASTed with other strains in GenBank database at E.value 0.00 and the results were showed that the query sequences were matched the *accD3* sequences in the databases with Query cover 100% and identity percentage 99%. **Table 3** shows the results of BLAST program (nucleotide Blast) for the eight obtained *accD3* sequences which were designated as (ALQM1, ALQM2, ALQM3, ALQM4, ALQM5, ALQM6, ALQM7, ALQM8)

TABLE 3: BLASTING RESULTS OF *accD3* SEQUENCES

Strain No.	E value	Identity (100%)	Max Score, Total Score	Query Cover(%)	Accession No. of similarities strains and Range
ALQM1	0.0	99	2710, 2710	100	HG813240.1 3042536 to 3044023
ALQM2	0.0	99	2732, 2732	100	CP009100.1 1006688 to 1008175
ALQM3	0.0	99	2732, 2732	100	CP007299.1 1004529 to 1006016
ALQM4	0.0	99	2693, 2693	100	CP007027.1 1006696 to 1008183
ALQM5	0.0	99	2473, 2473	99	AP014573.1 1010903 to 1012244
ALQM6	0.0	99	2405, 2405	99	CP009480.1 1003854 to 1005193
ALQM7	0.0	99	2416, 2416	99	CP009480.1 1003854 to 1005194
ALQM8	0.0	99	2447, 1447	98	HG813240.1 1005257 to 1006587

The whole sequences of *accD3* of different strains of this study (ALQM1, ALQM2, ALQM3, ALQM4, ALQM5, ALQM6, ALQM7, ALQM8) were deposited at NCBI with Accession numbers (LC006979, LC008196, LC009412, LC009414, LC034168, LC038020, LC041163, LC041368) respectively.

The eight nucleotide sequences were translated in to amino acids (495 amino acids) sequences using EMBOSS software (http://www.ebi.ac.uk/Tools/emboss/) and the resulted protein sequences were subjected for confirmation, then the eight protein sequences modeled by using SWISS-MODEL server (http://swissmodel.expasy.org/) [13] with three templates 2f9y, 2f9i and 1xou proteins, **Fig. 5** shows the best model for *accD3* model.

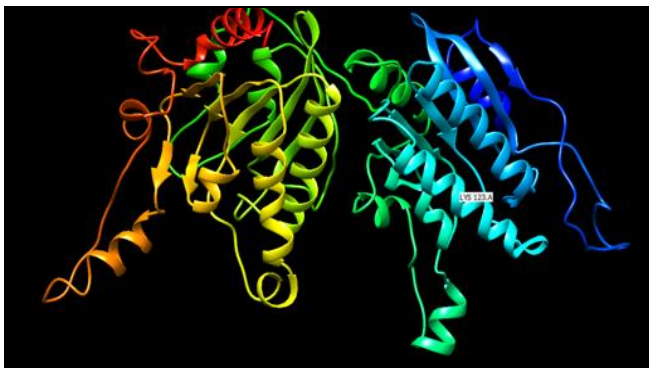


FIG. 5: *AccD3* PROTEIN (H37Rv STRAIN) 3D MODEL OF *M. TUBERCULOSIS*

All old and new drugs which were used in TB treatment and some natural products were collected from literatures and research papers and used as ligands in this study. These ligands are:

I. Old drugs: [33, 34]

Isoniazid (INH)
Rifampin (RIF)
Fluoroquinolone
Pyrazinamide (PZA)
Ethambutol (EMB)
Streptomycin (SM)
Cycloserine
Capreomycin
ρ-Aminosalicylic acid
Levofloxacin
Moxifloxacin
Gatifloxacin
Amikacin/Kanamycin
Ethionamide
Macrolides

II. New Drugs: [35]

Nitroimidazoles
PA-824
OPC-67683
TMC207
Linezolid

III. Natural products: [36]

Pyridomycin
Vitamin D3

Docking: Many softwares are available to predict the molecular interactions that may occur between a target protein and small molecule. Among these Swiss Dock, which is a web server dedicated for docking of small molecules on target proteins [37].

All 22 molecules and models of this study were entered to EADockDSS engine in the Swiss Dock server and the results were visualized with UCSF Chimera. Five molecules have a high negative free binding energy (lowest binding energy) and these were chosen for pharmacophore building [38].

TABLE 4: MOLECULES BINDING ENERGY

Molecules	Binding energy
Cycloserine	-11.24
Fluoroquinolone	-1.9
ρ-Aminosalicylic acid	-10.25
Vit.D3	-2.31
Ethambutol	-6.83

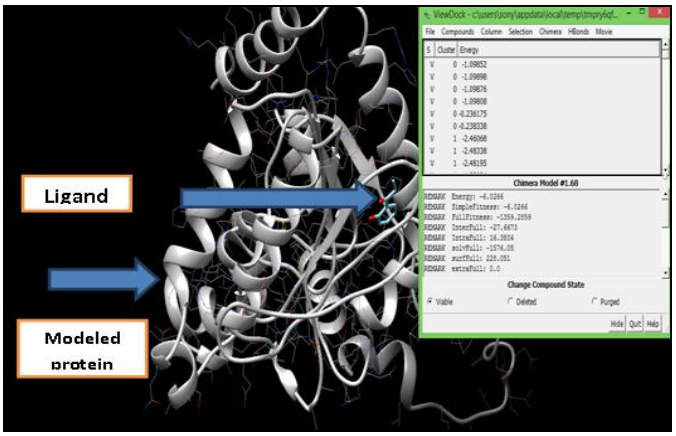
The characterization of the structure and the energetics of molecular complexes are a key factor for understanding biological functions and the energetics often provides the most important and useful link between structure and function of biomolecular systems. Furthermore, the prediction and design of ligands that can reversibly bind to pharmaceutical targets (enzyme inhibitors, receptor agonists and antagonists etc.) is at the heart of structure-based drug design. To be able to predict the strength of noncovalent associations, as well as the structures of molecular complexes, has therefore been an important objective in computational chemistry [39].

Virtual screening (VS) is the process of evaluating a library of compounds using a computational model in order to rank, and thus screen for molecules that exhibit desired characteristics, LBVS (Ligand based virtual screening) was adopted in this study and pharmacophore model generated from a set of known ligands which was used in the virtual screening to elicit specific inhibitors against *accD3* protein [40, 41].

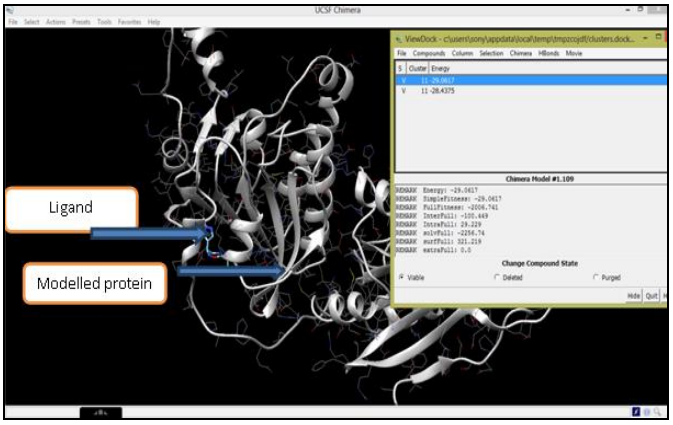
In this study, Discovery Studio program [42] was used for pharmacophore modeling and the five molecules from the previous step were entered to the Hip Hop generator at Discovery Studio program and gave two hypothetical pharmacophores (pictures not shown).

These pharmacophores were used in the Zinc Pharmer program (pictures not shown). Zinc Pharmer provides tools for constructing and refining pharmacophore hypotheses directly from molecular structure [43]. One thousand molecules were obtained from Zinc database after filtering through Lipinskie's rules of five, All molecules tested for their mutagenicity and toxicity with LAZAR and T.E.S.T softwares.

All molecules were used in the Swiss Dock to estimate their binding affinity to the proteins of all nine strain depending on the binding free energy **Fig.** (**6A, B**) shows the protein-ligand docking results with minimum binding energy visualized with chimera UCSF program (Data table not shown)



A



B

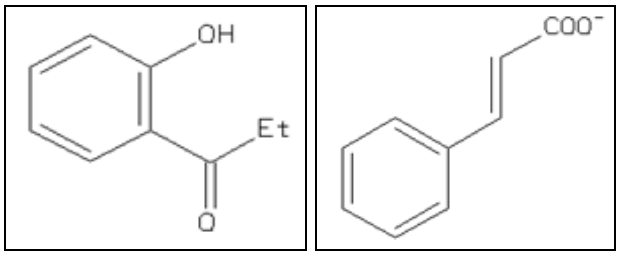
FIG. 6 A, B: PROTEIN-LIGAND DOCKING RESULTS

For *accD3* modeled protein (H37Rv strain), All molecules gave a negative binding energy (positive docking results) ranged between -2.3 as a lowest value and -36.7 as a highest value. Twenty nine molecules gave a negative binding energy with *accD3* modeled protein (ALQM1

strain) ranged between -1.15 as a lowest value and - 43.6 as a highest value. The same molecules gave a negative binding energy with the model of ALQM2 strain with -2.3 as a lowest value and -46.3 as a highest value. Twenty seven out of 30 gave a negative binding energy with the modeled protein of ALQM3 strain and values ranged between -1.4 as a lowest value and -43.5 as a highest value. Twenty nine out of 30 gave a negative binding energy with the modeled protein of ALQM4 ranged between -0.1 as a lowest value and -43.5 as a highest value.

All 30 molecules gave a negative binding energy with the model of ALQM5 strain and the value ranged between -3.34 as a lowest value and -42.08 as a highest value. Twenty seven out of 30 molecules gave a negative binding energy with the modeled protein of ALQM6 and the values ranged between -1.45 as a lowest value and -43.9 as a highest value. The same molecules gave a negative binding energy with the modeled protein of ALQM7 strain and the values ranged between -0.6 as a lowest value and -43.7 as a highest value. All molecules gave a negative binding energy with the modeled protein of ALQM8 and the values ranged between -1.9 as a lowest value and -38.8 as a highest value.

Two molecules with Zinc ID (391093, 16051516) are commercial available, the chemical structures of these molecules are shown in **Fig.** (**7 A, B**).

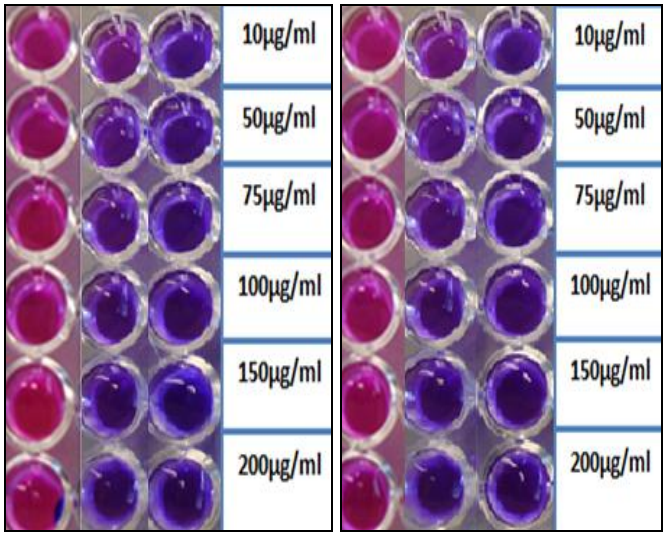


2'Hydroxypropiophenone **trans-Cinnamic acid**

FIG. 7: A, B: THE CHEMICAL STRUCTURES OF THE SELECTED LIGAND MOLECULES

***In vitro* Application of Drugs:** In this study, Resazurin Microtiter Assay Plate (REMA) was used to prove the inhibitory activity of ligands against the viable cells of different strains of *M. tuberculosis*, this test based on Resazurin which is a blue - purple, non-toxic, oxidation-reduction indicator that becomes pink when reduced to resorufin by cellular oxidoreductases [44]. This assay is colorimetric and the rate of color change is directly proportional to the number of viable cells in the initial suspension. In other words, the concentration of viable cells in a suspension containing resazurin directly determines the visible conversion from purple to a pink color [44, 45].

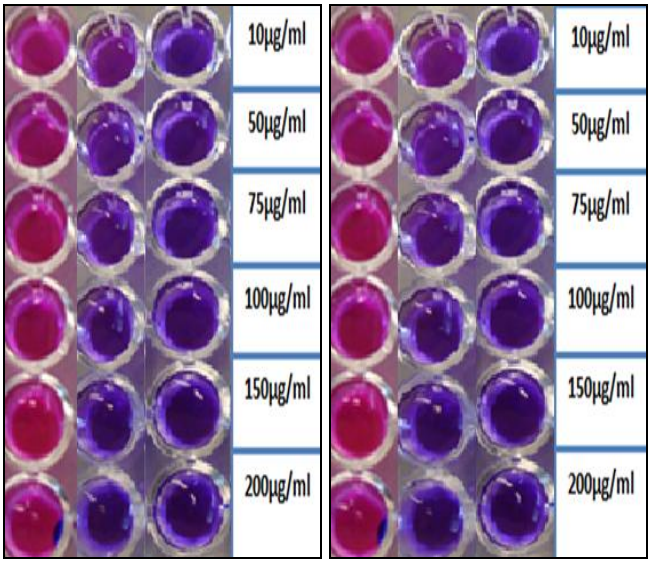
The wells with dark purple color are more sensitive to the added molecule suspension when compared with wells of the control, and this match with the study of [45], in this study, six different concentrations (10 µg/ml, 50 µg/ml, 75 µg/ml, 100 µg/ml, 150µg/ml, 200µg/ml) of Ligand I (trans-Cinnamic acid), Ligand II (2'-Hydroxypropiophenone) and Ligand III (as a control) (4-Amino-2-hydroxybenzoic acid) were applied on sensitive and MDR strains of *M. tuberculosis* and the inhibitory activity of these ligands determined depending on color change from blue - purple (resazurin color) to pink color as shown in **Fig. 8** for ligand 1 which have been inspect visually the effect on two sensitive strains of *M. tuberculosis* (sensitive to the routine drugs regime in TB treatment), the results described as high sensitive to the new ligands at concentration above 10µg/ml.



A (Sensitive I) **B (Sensitive II)**

FIG. 8: EFFECT OF LIGAND MOLECULE I (TRANS-CINNAMIC ACID) ON TWO DIFFERENT SENSITIVE STRAINS (I, II), FROM LEFT TO RIGHT, POSITIVE CONTROL, LIGAND1, NEGATIVE CONTROL

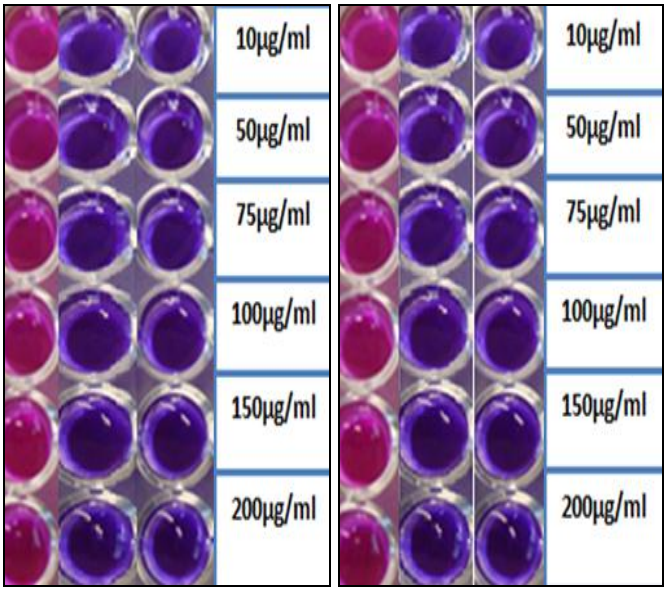
Fig. 9 shows the effect of ligand I on two MDR strains of *M. tuberculosis*, the results described as high sensitive to the new ligands at concentration above 75µg/ml.



A (MDR I) **B (MDR II)**

FIG. 9: EFFECT OF LIGAND MOLECULE I (TRANS-CINNAMIC ACID) ON TWO DIFFERENT MDR STRAINS (I, II), FROM LEFT TO RIGHT: POSITIVE CONTROL, LIGAND1, NEGATIVE CONTROL

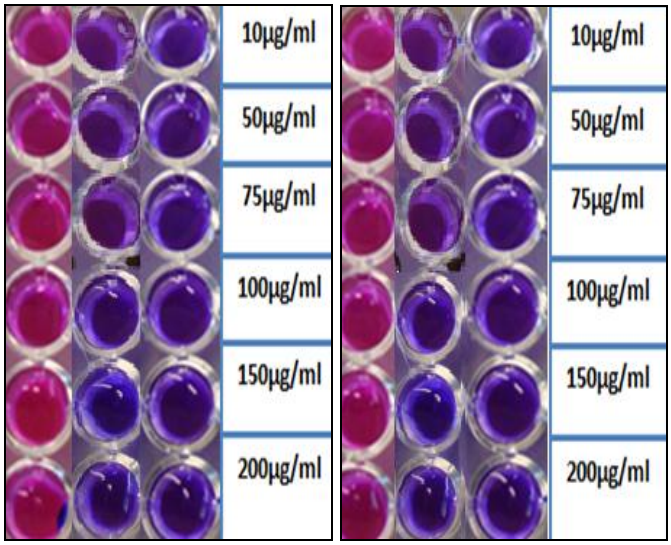
Fig. 10 shows the effect of ligand II on two sensitive strains of *M. tuberculosis* and the results described as high sensitive at all concentrations.



A (sensitive I) **B (Sensitive II)**

FIG. 10: EFFECT OF LIGAND MOLECULE II (2'-HYDROXYPROPIOPHENONE) ON TWO DIFFERENT SENSITIVE STRAINS (I, II), FROM LEFT TO RIGHT: POSITIVE CONTROL, LIGAND II, NEGATIVE CONTROL

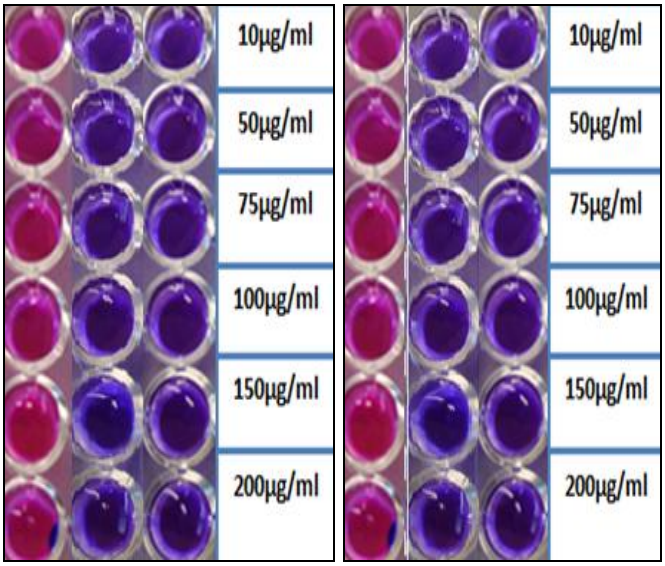
Fig. 11 shows the effect of ligand II on two MDR strains of *M. tuberculosis* and the results described as high sensitive at concentration above 75µg/ml.



A (MDR I) **B (MDR II)**

FIG. 11: EFFECT OF LIGAND MOLECULE II (2'-HYDROXYPROPIOPHENONE) ON TWO DIFFERENT MDR STRAINS (I, II), FROM LEFT TO RIGHT: POSITIVE CONTROL, LIGANDII, NEGATIVE CONTROL

Fig. 12 shows the effect of ligand III on two different sensitive strains of *M. tuberculosis* and the results described as high sensitive at all concentrations.



A (Sensitive I) **B (Sensitive II)**

FIG. 12: EFFECT OF LIGAND MOLECULE III (4-AMINO-2-HYDROXYBENZOIC ACID) AS A CONTROL ON TWO DIFFERENT SENSITIVE STRAINS (I, II), FROM LEFT TO RIGHT: POSITIVE CONTROL, LIGAND III, NEGATIVE CONTROL

From *in vitro* studies, it could concluded that:
Sensitive isolate: Trans-Cinnamic acid is effective at 100µg/ml, 150µg/ml & 200µg/ml.

It is obtained from oil of cinnamon, it is also found in shea butter. [46] used trans - cinnamic acid as anti-hepatitis virus agent.

2'-Hydroxypropiophenone is effective at all concentrations. It is used as a flavorant to enhance the flavor of wintergreen-flavored products [47].

4-Amino-2-hydroxybenzoic acid is effective at all concentrations.

MDR isolate:
Trans-Cinnamic acid is very effective at 150µg/ml, 200µg/ml.

2'-Hydroxypropiophenone is very effective at all concentration.

The difference of effects attributes to the variations of sensitive strains, and due to the changes occurred in mutated strains, looking for the real reasons need more studies at least at the molecular level. It might be expected to be something related to permeability and pump effluxes.

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