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Uropathogenic *Escherichia coli* Antibiotic Resistance and *in silico* Virtual Screening Using Pharmit Technique

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Abstract: Increasing the rate of antibiotic-resistant pathogens causing serious infections and death has become a global health threat. However, the synthesis of antibiotics and new drugs is expensive and undertaken through multi phases and clinical trials. Therefore, repurposing old drugs to treat antibiotic-resistant bacteria is a good alternative way. *Escherichia coli* (*E.coli*) is a common pathogen of urinary tract infection, and its capability to form bifilms further contributes to its virulence and antibiotic resistance, posing a big crisis to global public health. Uropathogenic *E.coli* were isolated then tested by Vitek2 and disk diffusion method to know which antibiotics are resistant to *E.coli*. Then, real-time PCR, conventional PCR, and genes sequences tools were used to identify the strain of isolates *E.coli*. To do repurposing antibacterial drugs or to find a new compound, we performed a virtual screening on Pharrmit provides an online, interactive environment for the virtual screening of large compound databases using pharmacophores, molecular shape, and energy minimization to predict a compound that has biological activity against the uropathogenic E. coli. A total of 14678 small molecules from the Drug Bank database were subjected to screening via Pharmit. Nine potential hits were obtained (100009383 - 1531009). Molecular docking can be used to evaluate the affinity of the new compounds to predict the binding affinity to *FimH* receptor.

Keywords: Uropathogenic, Virtual Screening, Pharmit.

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Introduction

Urinary tract infections (UTIs) are related to high rates of disease and mortality worldwide (1, 2). Since about 150 million people worldwide are diagnosed with UTI every year, therefore the diagnosis and treatment of UTIs are the main concern in the field of healthcare (1, 3). UTIs are caused by a range of different pathogens but the most frequent causative factors for UTIs are uropathogenic *Escherichia coli* (UPEC) strains (4). The Gram-negative bacteria, the UPEC accounts for 50-85% of nosocomial and the main etiologic factor responsible for UTIs, which are classified according to the site of infection: urine (asymptomatic bacteriuria), bladder (cystitis), kidney (pyelonephritis) and blood (urosepsis and bacteremia) (5, 6). UPEC strains isolated from the urinary

tract are known as UPECs (7). The UTIs caused by the *E.coli* strain include a large range of disorders, including cystitis, urethritis and pyelonephritis. The frequency of this infection is 1% in males and 3-8% in females (8). *E.coli* strains derive from different phylogenetic groups; phylogenetic typing in four groups: A, B1, B2 and D. The majority of strains responsible for UTIs belong to B2 and D groups (9, 10). To know the mechanism underlying the pathogenicity of *E.coli*, a vital number of virulence-related genes or functions associated with different stages of infection have been identified (11). UPEC strains possess an arsenal of virulence factors that contribute to their ability to overcome different defiance mechanisms that cause disease. These virulence factors that are located in virulence genes include fimbriae which help bacterial adherence and invasion, iron-acquisition systems, flagella and toxins (12). UPEC express a few hundreds of these organelles on their cell surface to adhere in a multivalent fashion to the superficial bladder cells. Adhesion is mediated at the molecular level by *FimH* binding to highly-mannosylated glycoproteins (MGP). Therefore, there is a dire need for new therapeutic approaches to eliminating pathogenic *E.coli* infections. One such therapeutic approach is to target the virulence factors involved in the bacterial adhesion of UPEC to the urothelial surface. Rather than aiming to kill the bacteria, as is the focus of common UTI antibiotics, a novel anti-adhesive approach that can be a suitable treatment method to eliminate UPEC infection (13, 14). There are a lot of software programs and websites that help with computer-aided drug creation (15), but there aren't many that offer structure-based virtual screening. Those that do exist, such as DockBlaster (16) and iDrug, are examples (17). Pharmit can elucidate pharmacophore and shape queries using receptor and/or ligand structures, or it can use a predetermined pharmacophore query as input. Structures can be supplied by the user or directly derived from the Protein Data Bank (PDB) (18). This study aimed to design and generate *In-silico* anti-adhesion against *FimH*.

Materials and Methods

Isolation of *Escherichia coli*

At the laboratory of the Al Karama hospital Wasit, Iraq under aseptic conditions, the collected specimens were streaked directly on MacConkey and Eosin methylene blue (EMB) agars then incubated for 24hours at 37°C under aerobic conditions. The lactose fermenting colonies on MacConkey agar were subculture on another MacConkey and EMB agar plate and incubated for another 24 hours at 37°C and Lactose-fermenting produces a dark purple complex usually associated with a green metallic sheen on EMB and pink colonization on MacConkey. Further identification tests included the morphological characteristics and biochemical tests were carried out (3).

Bacterial Strains and Culture Conditions

Uropathogenic *E.coli* were isolated from the urine of a UTI patient at Al Karama Hospital Waist, Iraq. *E.coli* ATCC 25922 (Iranian biological resource centre) and Uropathogenic *E.coli* were

typically cultured in MacConkey agar (Himedia India)then cultured in the Tryptic Soy Broth (TSB) (Thermo fisher scientific: USA) and incubated for 24 h at 37 C° and stocked at -20 C° with 20% glycerol. Then, the colonies were suspended in 5 mL of TSB in a 50 ml Falcon tube (Greiner Bio-One, Kremsmünster, Austria) and grown to exponential phase under aerobic conditions at 37 C° with shaking (220 rpm), until the culture reached a required concentration based on McFarland test (the culture was diluted to 1×10^6 CFU mL−1 before starting the experiment). Later the Vitk2 was used to determine the antibiotic resistance of *E.coli*. After that, colonies were directly used to start the experiment.

Total RNA Extraction

RNA was extracted from five different *E.coli* strains were obtained from hospital samples isolated from patients suffering from urinary infections. The bacteria were grown in a cultured medium (MacConkey) specifically for *E.coli* species at 37 °C overnight. The total RNA content was extracted as follows:

The fresh grown bacteria cells were precipitated by centrifugation at 3000 × g for 10 minutes. Then, the supernatant was removed and 1 ml Kiazol reagent (kiazist Co., Iran) was added to each 1 × 107 bacterial cells. After that, the cells were completely lysed by repetitive pipetting and incubated at room temperature for 5 minutes to ensure the complete dissociation of nucleoprotein complexes. For each 1 ml Kiazol reagent initially used, 0.2 ml chloroform was added to the cell lysate and vigorously shacked for 15 seconds. The resulting solution was incubated at room temperature for 10 minutes. To separate the solution into three phases, microtubes were centrifuged at 8000 × g for 20 minutes at +4 °C. After centrifugation, the colourless upper aqueous phase containing RNA content was carefully transferred into a new microtube. To Precipitate the RNA content from the colourless aqueous phase of each sample, 0.5 ml isopropanol was added for each 1 ml Kiazol reagent used in the initial step. The tubes containing RNA content and isopropanol were capped and inverted twice to be mixed thoroughly. The samples were incubated for 5 minutes at room temperature to allow the RNA precipitate to form. To precipitate RNA content, the solution was centrifuged at 8000 × g for 15 min at +4 °C. After centrifugation, the supernatant was discarded and 0.5 ml 75% ethanol was added to each sample. The RNA pellet of each sample was washed in 75% ethanol by vortexing for 5-10 seconds and was then centrifuged at 7500 × g for 5 minutes at +4 °C. Then the supernatant was discarded and the tubes were kept upside down on a clean tissue paper to remove extra ethanol and air-dry the RNA pellet. The air-dried RNA pellet of each sample was resuspended in RNase-free water and incubated at +55 °C for 5 minutes to dissolve the RNA pellet. Finally, the RNA samples were stored at -20 °C until used.

cDNA synthesis

The synthesis of DNA from an RNA template, via reverse transcription process, produces complementary DNA (cDNA). Reverse transcriptase (RT) enzymes use an RNA template, dNTPs, and a short primer complementary to the RNA template to direct the synthesis of the first-strand cDNA, which can be used directly as a template for the Polymerase Chain Reaction (PCR). In this study, the Easy^{TM} cDNA synthesis kit (Parstous Co., Iran) was provided for synthesizing the cDNAs from the extracted total RNAs of five *E.coli* strains.

cDNA synthesis conditions

According to Table 1, all components needed for cDNA synthesis were combined in a sterile microtube, mixed gently, and incubated in a thermal cycle running the following thermal program demonstrated in Table 2. All cDNA samples were stored at -20 °C until used.

Table 1. The components of the cDNA synthesis reaction

Component	Amount
RT buffer mix + Enzyme mix	5 µl
Primers (Oligo d(t) and random hexamer)	1 µl
Nuclease free water	2 µl
Total RNA (□1 µg/ µl)	2 µl
Total volume	10 µl

Table 2. The thermal program for cDNA synthesis

cDNA synthesis program	
Initial preparation	25 °C for 10 min
cDNA polymerization	47 °C for 60 min
RT enzyme inactivation	85 °C for 5 min

Primers Design, Selection and Preparation

In this work, the expression levels of three *fimH, papG and csgA* genes were examined by real-time PCR in five *E.coli* strains. The specific primers for each *fimH, papG* and *csgA* gene were designed using Oligo7 software and their efficiency and specificity were checked using NCBI primer-blast and online oligo analyzer tools. The 16Sr RNA gene was used as the reference gene. The list of primer sequences was provided in Table 3.

Table 3. Listed the Sequences of the Primers Used for the primer sequences used for real-time PCR *16S RNA, fimH, papG and csgA Genes.*

Gene name		Sequence (5'-3')	Amplicon size (bp)	Tm	Length
fimH	(F)	TTACTCTGGCGGACTACCCTG	138	60	21
	(R)	AAAACGAGGCGGTATTGGTGA		60	21
papG	(F)	AGGTAACGGGTAGCGAAGGTG	110	61	21
	(R)	CTCTTCGTGACCGTGCTGTTT		61	21
csgA	(F)	CTCCAAACTGATGCCCGTAACT	132	60	22
	(R)	TGTAGCCCTGTTACCGAAGCC		62	21
16S rRNA	(F)	ACTCCTACGGGAGGCAGCAG	197	63	20
	(R)	ATTACCGCGGCTGCTGG		60	17

Real-time PCR procedure

To evaluate the gene expression of *fimH*, *papG* and *csgA* in all five *E.coli* strains, the real-time PCR components were combined in a sterile PCR microtube as shown in Table 4. All added materials were gently mixed with cDNA and finally, the PCR was run according to the thermal program provided in (Table 5).

Table 4. The components of a real-time PCR reaction

Components	Amount
Add SYBR mix (2X Conc.)	5 µl
Primer mix (F, R) (10 µM)	1 µl
Distilled water	3 µl
cDNA	1 µl
Total volume	10 µl

Table 5. The condition of Real-time PCR.

Steps	
Initial denaturation	95 ℃, 5 min
PCR cycling (40 cycles)	95 ℃, 30 sec 58 ℃, 30 sec 72 ℃, 30 sec
Melting analysis	95 ℃, 5 min 60 ℃, 1 min Raising from 65 ℃ to 90 ℃ at a rate of 0.3 ℃

The expression level of each gene in different strains was calculated by using a $2^{-\Delta Ct}$ formula, where Δ CT (threshold of a cycle) of each gene was obtained by subtracting the Ct of each gene from the CT of the reference gene. The analyzed results of each gene were illustrated as a graph using graph pad prism software (version 9).

Conventional PCR and sequencing

To obtain the entire sequence of *fimH*, *papG* and *csgA* genes of the selected *E.coli* strain and confirm their sequence, the conventional PCR and sequencing techniques were performed respectively.

Primers Design, Selection and Preparation

For conventional PCR, a new set of primers was designed to amplify the whole genes by PCR (Table 6). The PCR mixtures for each gene was prepared as described in Table 7 and the PCR program was run according to the thermal program provided in (Table 8).

Table 6. Listed the Sequences of the Primers Used for the primer sequences used for conventional PCR to Detect *Escherichia coli fimH, papG* and *csgA genes.*

Gene name	Gene name	Sequence (5'-3')	Amplicon size	Tm	Length
fimH	(F)	GCAGCCACTCAGGGAACCA	1090	62	19
	(R)	TAAGCCAGATGCGACGCTGAC		63	21
papG	(F)	GGCATCATGGAAGGCGAATAC	595	59	20
	(R)	CACGCGCAAAAAAAAAGCCAG		60	21
csgA	(F)	GTAGAGCAGAGACAGTCGCAA	635	60	21
	(R)	GGAAAGTGCCGCAAGGAGTAA		61	21

Table 7. The components of conventional PCR

Components	Amount
2X Taq master mix	5 µl
Primer mix (F, R) (10 µM)	1 µl
Distilled water	3 µl
cDNA	1 µl
Total volume	10 µl

Table 8. Conventional PCR program and condition

Steps	
Initial denaturation	95 ℃, 5 min
Amplification (40 cycles)	95 ℃, 30 sec 58 ℃, 30 sec 72 ℃, 1 min
Final extension	72 ℃, 5 min
Final hold	4 ℃, 5 min

After PCR, the agarose gel electrophoresis was performed to observe the PCR products. To do this, 1% agarose gel in 1X Tris-acetate-EDTA (TAE) buffer was prepared, the PCR products were loaded in agarose gel and electrophoresed at 70 voltages for 20 min. the DNA bands were visualized under a UV laminator.

Antimicrobial susceptibility testing - Disk Diffusion Method (DDM)

Antimicrobial susceptibility testing was performed to examine the antibiotic resistance rate, susceptibility testing was prepared according to (19) as follows:

- Colonies from an overnight nutrient agar plate culture were transferred to 3ml of normal saline. the turbidity was adjusted to 0.5 McFarland equal to 1.5×10^8 CFU/ml by using DensiChek.
- A sterile cotton swab was dipped into the bacterial suspension; excess fluid was removed by pressing the swab against the tube wall. The bacterial suspension was inoculated into Muller Hinton agar plates and the plates were left to dry for 10 minutes.
- Different antibiotic discs were used with a maximum of eight discs were placed on the surface of the medium using sterilized forceps then incubated at 37°C for 24 hrs.
- When the incubation was complete, the resulting zones of inhibition were measured and compared with the breakpoints of Clinical Laboratory Standards Institute CLSI(20).

Pharmacophore Method
From receptor/ligand files

Alternatively, the user may first choose "enter pharmit search" to upload structural files. After being redirected to the search page, choose "Load Features" to upload a small molecule structure in SDF, PDB, mol2, or XYZ format. A receptor file in any of the same formats may optionally be provided as well; our example uses both the ligand and receptor. To initiate the 4pps search using this method, the user should load the attached receptor and ligand files as the receptor and features, respectively. These files were obtained directly from the 4pps PDB entry and are provided as examples of possible input formats. If a receptor is provided via the "Load Receptor" option, it should be one for which the binding pose of the provided ligand is known, or to which the ligand was previously docked. pharmit will not dock the two

compounds, and since the receptor is used to identify which pharmacophore features of the ligand are relevant to binding, providing a pair of structures that are oriented arbitrarily will fail to identify a relevant pharmacophore. If a ligand structure is provided without a receptor, the query will proceed normally but the user will not be able to energy minimize the results of queries since this calculation depends on the presence of a receptor. The second image below shows an example of uploading a ligand structure without a receptor (18).

Results and discussion

Phenotypic identification

In the approach of this study, five clinical samples were collected from urine female patients at AL Karama hospital, Waist Governorate, Iraq. Those samples were collected at (30- 40) years of age. All specimens were cultured on a selective medium such as MacConkey agar and Eosin Methylene Blue (EMB) agar. The isolates obtained from those media were identified according to the following characters, observed.

MacConkey agar Medium

MacConkey agar plate the recorded observations here on the growth and appearance of the bacteria growth on MacConkey agar, the organism on this plat is an *E.coli* lactose fermenter, as evidenced by the pink colour.

Eosin Methylene Blue (EMB) agar

EMB agar plate the recorded observations here on the growth and appearance of the bacteria growth on Eosin Methylene Blue Agar, the organism on this plat is an *E.coli* lactose fermenter, as evidenced by produces a green metallic sheen. The most common and widespread detection methods include characterization via a phenotypic system and commercial phenotypic methods (e.g., the Vitek-2 system [Biomerieux]. which have been used to successfully identify most Acinetobacter species. However, there are some limitations to these methods (21).To identify the expression level of target *FimH* of the isolated uropathogenic *E.coli* strains from five patients, it's important to use RT-PCR and conventional PCR. The results showed that *FimH* was high levels of expression in strain 4 (S4) while other strains were low levels in expression (Figure 1. a).

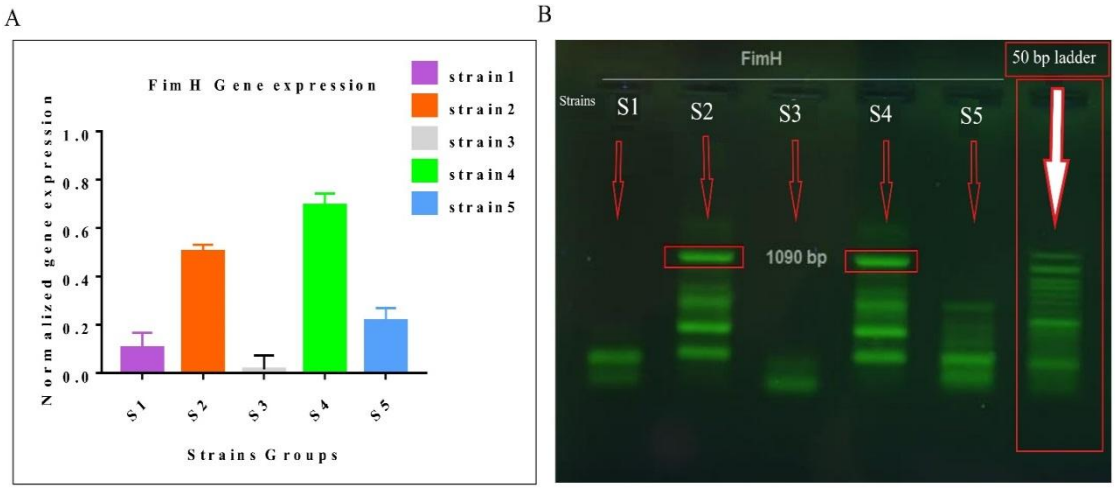

Figure 1: Expression levels (value assessed by real-time PCR) of *FimH* gene.
A) shows the levels of expression of *FimH* gene in uropathogenic was high in S4.
B) Gel electrophoresis gels show the bands of *FimH* gene was also thick in S4.

Evaluation of relative expression of FimH gene by real-time PCR (qPCR). According to qPCR data, strains 2 and 4 showed the FimH expression significantly higher than strains 1, 3, and 5. thus strains 2 and 4 could be considered as proper strains. DNA bands related to FimH PCR products were detected on the agarose gel. The correct band for FimH was a 1090 bp fragment which was observed only in strains 2 and 4 (Figure 1. b). The FimH-related 1090 bp fragment from strain number 4.

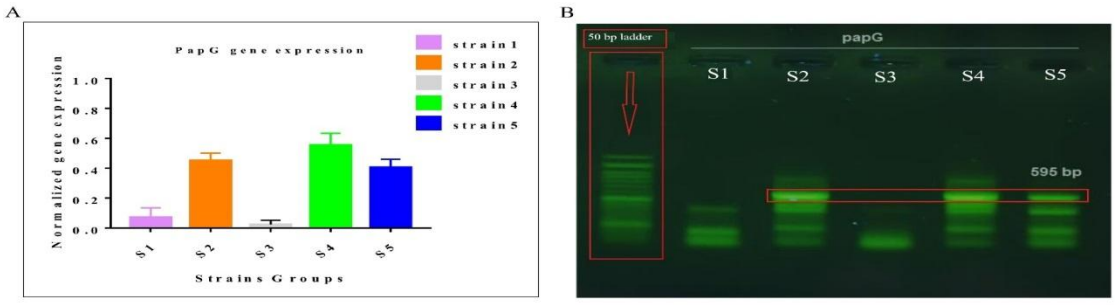

Figure 2: Expression levels (value assessed by real-time PCR) of *papG* gene. A) shows the levels of expression of *papG* gene in uropathogenic was high in S4. B) Gel electrophoresis gels show the bands of *papG* gene was also thick in S4.

Evaluation of relative expression of papG gene by real-time PCR (qPCR). The qPCR data exhibited that all five strains expressed papG gene. However, strains 1, 2, and 3 showed low expression of papG, strains 4 and 5 showed significantly high expression of papG (Figure 2. a). Therefore, it was concluded

that strains 4 and 5 expressing papG gene at a high level seemed to be suitable for further examinations on papG gene. DNA bands were observed after electrophoresis of papG PCR products. The band related to papG was detected as a 595 bp fragment which was present in strains 2, 4, and 5 (Figure 2. b). The papG -specific 595 bp fragment from strain number 4.

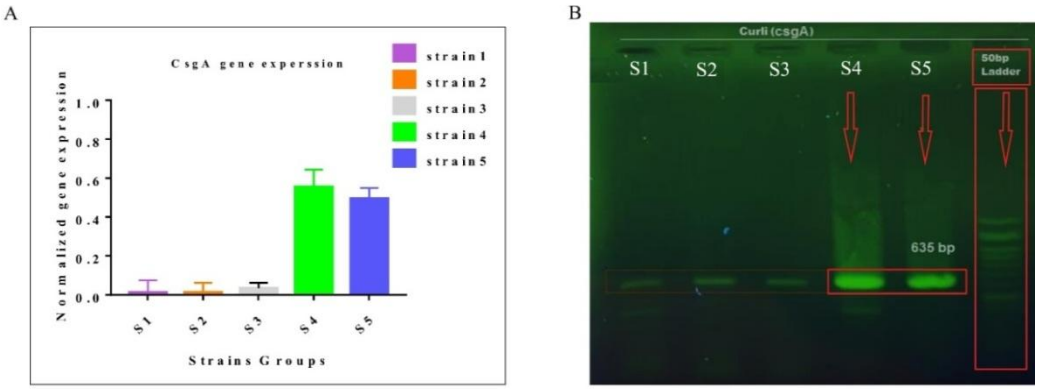

Figure 3: Expression levels (value assessed by real-time PCR) of the curli gene. A) shows the levels of expression of the curli gene in uropathogenic was high in S4. B) Gel electrophoresis gels show the bands of the curli gene was also thick in S4.

Evaluation of relative expression of csgA gene by RT-PCR (qPCR). The qPCR data showed that among five selected strains, strains 4 and 5 exhibited high expression of csgA gene and the expression level of csgA gene was quite low in strains 1, 2, and 3. these results suggested that strains 4, and 5 could be proper strains for following studies on csgA gene (Figure 3. a). The correct DNA band related to csgA gene (635 bp) was detected in all five strains. Among them, strains 4 and 5 showed a sharper band, indicating a higher-level expression of csgA gene and the expression level of csgA gene (Figure 3. b).

Antibacterial Susceptibility Test

ATCC 25922 (Iranian biological resource center) and Uropathogenic of strain 4 E.coli bacteria have been identified and subjected to antibiotics to check their resistance as following antibiotics: Ampicillin B(AM), Azithromycin (AZM), Ciprofloxacin (CP), Cefpodoxime (CV), Gentamicin (GM), Trimethoprim (SXT), Norfloxacin (NOR), Streptomycin(S), (Table 9). The results demonstrate the bacterial resistance of uropathogenic when subjected to eight antibiotics, two of them were not resistant to Cefpodoxime (CV), Gentamicin (GM). In contrast to the other antibiotics were showing no activity to stop the growth of the pathogenic bacteria Figure(4).

Table (9): antibiotic Susceptibility of ATCC and Pathogenic *E.coli.*

	Antibiotics	cod	Antibiotics resistance	
			ATCC – Bacteria	Pathogenic – *E.coli*
1	Ampicillin	AM	S	R
2	Azithromycin	AZM	S	R
3	Ciprofloxacin	CP	S	R
4	Cefpodoxime	CV	S	S
5	Gentamicin	GM	S	S
6	Trimethoprim	SXT	S	R
7	Norfloxacin	NOR	S	R
8	Streptomycin	S	S	R

R: Resistance, S: Sensitive.

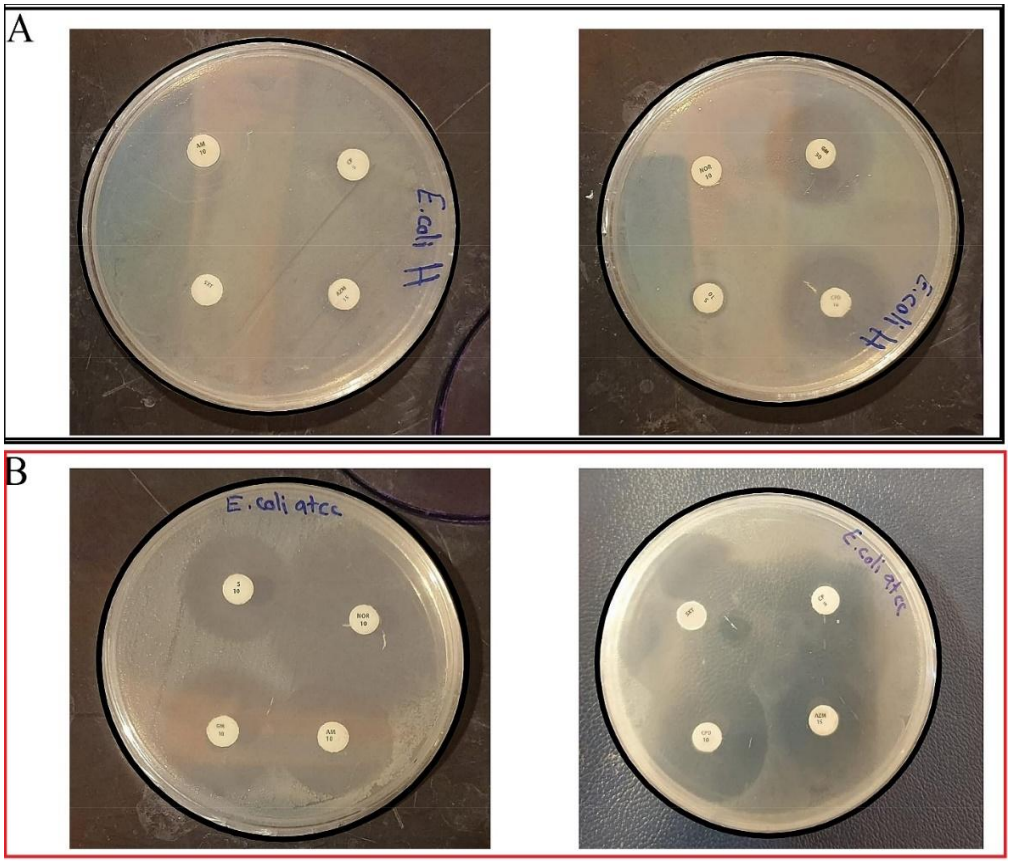

Figure 4: Antibiotics Susceptibility of UroPathogenic *E.coli* and reference strain. A) Antibiotics Susceptibility for Uropathogenic *E.coli.* B) Antibiotics Susceptibility for reference bacteria.

Furthermore, the two antibiotics which have the biological activity to reduce the growth of the pathogenic bacteria (Table 9), Disc diffusion methods were used in this study to Show antibiotic resistance for Pathogenic and ATCC Bacteria. Furthermore, Cefpodoxime is one of the active antibiotics that could be used as a loading feature in pharmacophore modelling to inhibit or reduce the growth of pathogenic bacteria. This result was in agreement with Aljanaby (22), who showed that *Escherichia coli* isolates were resistant to the most used antimicrobials in high percentages.Also, the results of this study agreed with Al-Kadmy (23), who revealed that the ability to survive in hospital environments assisted *Escherichia coli* to emerge as one of the most successful nosocomial pathogens as 1st. the leading pathogen in the intensive care units due to its increasing multidrug resistance. These results indicated that uropathogenic *E. coli* is resistant to all antibiotics except Cefpodoxime and Gentamicin. Therefore, these two antibiotics were used to control with drugs that were used in this study.

Query Finding of Pharmit

The web server Pharmit provides two search modalities depending on whether the shape query is the primary query or the pharmacophore. If desired, the complete set of query-aligned results may be saved as a compressed SDF structure file. In both instances, the primary query determines the pose alignment of the hit compounds and secondary inquiry serves as an additional filter.

Pharmacophore Model

To identify bioactive molecules, the computational approach to drug development has a prominent significance. The structure-based virtual screening mainly considers the molecular docking method (24). A wide range of compound libraries of individual interest can be screened through virtual screening methods. As a result of pharmacophore at pharmit webserver (Figure 5), we have got 9 hits (100009383, 3812862, 100009383, 1531009, 1533877, 3803652, 1531008, 3812862 and 1531009) by screening the drug bank database, when the features of binding were H-doner-4, H-Acceptor-3 groups (Figure 6). Whereas after manipulating with features like the molecular weight between 200 up to 500, those manipulating could reduce the result of molecules number from 473 to 119 hits only (Figure 6). The most studied agents are manosides that block the binding of *FimH* to host receptors. *FimH* is the tip of the type 1 fimbriae of *E.coli* that mediates the first step in biofilm formation. Manosides appear to have a good protective role in urinary tract infections caused by *Escherichia coli* because they not only interfere with adherence but also enhance the effect of the antimicrobial agent cotrimoxazole (25). The protein complex KGM ligand was our chosen molecule to be loaded at pharmit, which would help to use its features against the target protein.

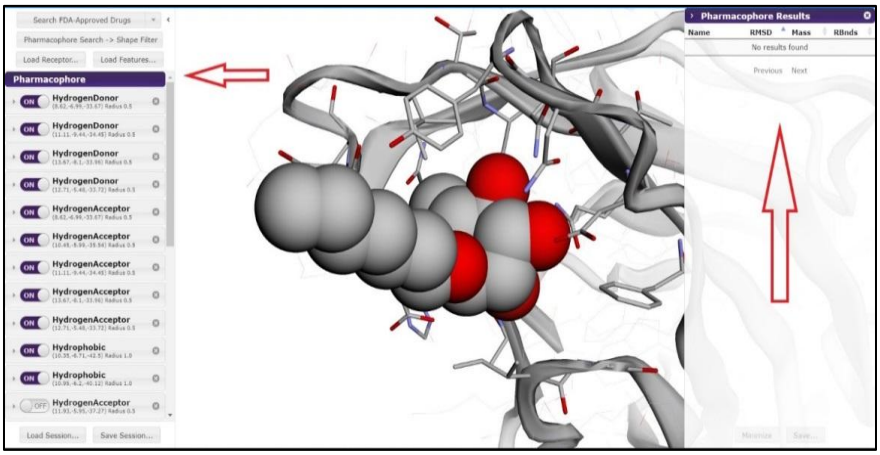

Figure 5: Pharmacophore first applies the receptor & load features to pharmit.

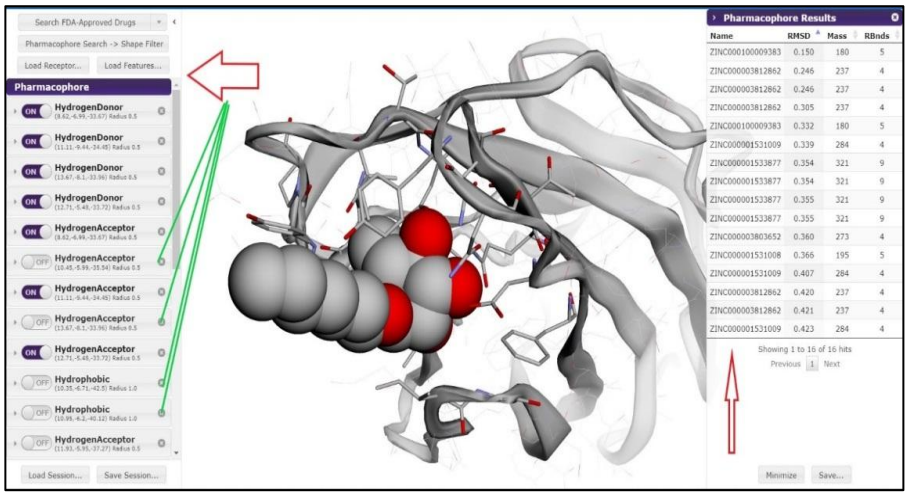

Figure 6: Pharmacophore features manipulating on pharmit

Virtual screening servers that may be accessed with a web browser. Its search speed, which is on the order of seconds; its high-resolution, smoothly animated visual interface; its customizability, which includes the ability to create custom compound libraries; and its comprehensive suite of search features, which includes an array of chemical constraints that allow the user to quickly identify hits that are also promising drug candidates, are just a few of its advantages. It democratizes structure-based computer-aided drug discovery by providing free and open access to cutting-edge chemical space exploration software, making it excellent for both research and education(18).

Physical chemistry educators can use a high-quality product to demonstrate essential ideas. The project can be modified to fit within existing drug discovery workflows. Pharmit's completely open-source nature allows it to be deployed locally by the user, ensuring complete privacy of queries and results in situations where intellectual property protection is a concern. Pharmit allows users to quickly and easily execute structure-based virtual screening.

Conclusions

With no doubt, pharmacophore modelling approaches are one of the most widely used and successful tools in Computer-aided drug design (CADD) projects and medicinal chemistry. Even though pharmacophore modelling has progressed significantly, more advancements in the application of pharmacophore modelling methodologies are required. Several elements, such as establishing accurate, optimized / predictive models, improved handling of ligand flexibility, and a deficient algorithm for ligand alignments, must be explored to improve its success rate in real-time studies. There may be a beneficial contribution in the form of target binding-site information, which can aid in the more efficient development of structure-based models. In the domain of fragment-based drug design, better methods involving pharmacophore fingerprint-based similarity search and production of 3D pharmacophore queries can be developed. As a result, the use of pharmacophore modelling approaches in the development of therapies opens up a slew of new possibilities. Furthermore, the results of this compound could be verified with molecular docking and dynamic simulation as the best inhibitor for the target protein receptor.

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