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Research Article

ANTIMICROBIAL ACTIVITY OF DIETHYL PTHALATE ISOLATED FROM MARINE STREPTOMYCES MW09-1: AN *INSILICO* APPROACH

V. Aruna

Department of Microbiology, Cauvery College for women (autonomous), Trichy, India

**Corresponding author: arunajai2012@gmail.com*

ABSTRACT

Marine actinomyces MW09-1 isolated from coastal area of athirampattinam, Thanjavur, Tamil nadu, India. The strain was identified as *stretomyces* species based on morphological, physiological, biochemical characters along with 16s rRNA analysis. The active extract was purified and the structure elucidated by GC-MS, NMR Spectra. It was identified as diethyl phthalate. Diethyl phthalate identified from the *stretomyces* strain showed significant antibacterial activity against uropathogenic *E.coli* with a zone of inhibition value of 16.6±2.08mm. Insilico Docking analysis was carried out by binding affinities between Diethyl phthalate, Ciproflaxcin and Intimin protein expressed by EAE gene. It showed that the compound had minimum binding energy of -25.3388 kg/ml and had a good affinity. Hence the diethyl phthalate could be an effective natural antimicrobial compound.

Keywords: Streptomyces, Diethyl phthalate, Ciproflaxcin, Intimin, *In silico* docking,

1. INTRODUCTION

Nature is the best source of all medicinal compounds and a number of modern drug have been isolated from natural environments, many based on their use initially in traditional medicine and later in the developed countries as single agent drugs on scientific bases. In addition to the health hazards of different origin the challenges of emerging and re-emerging diseases and the development of multidrug resistant infectious microorganisms to existing drugs and the urgent requirement of new drugs against the resistant pathogens are the live task to be addressed by the present day scientific community. Microorganism's especially marine actinomycetes are being examined as a source of novel antibiotics that are active against antibiotic resistant human pathogens [1]. Regarding secondary metabolite production from microorganisms isolated from the marine, it has been found to remain normally dormant or weakly expressed under laboratory condition which was difficult from prior to extensive chemical investigation [2].

Species of streptomyces as versatile producers of new secondary metabolites from different biosynthetic pathways originate from different ecological niches and could be used to search for antimicrobial components [3]. Antimicrobial compounds from marine streptomyces of unique structure and obvious effect have been obtained; however how to tap these treasurous compounds? (Li et al 2013) Researchers [4] suggested that streptomyces isolates from marine sediments are valuable for the production of bioactive compounds. Compounds synthesized by most strptomyces species with broad spectrum features, including antibiotics, pesticides, herbicides, enzyme inhibitors and commercially effective drugs, and occupy approximately one third of the known isolated metabolites from streptomyces [5].

Therefore, the purpose of this study is to evaluate, in vitro, the antagonistic activity of bioactive metabolites from marine *streptomyces sp.,* against virulent UTI pathogen *E.coli*. Moreover, the study would extend to separate, analyze and characterize the crude extract of antagonist using Gas chromatography- mass spectrometry (GC-MS) and NMR analysis.

2. MATERIAL AND METHODS

2.1. Isolation and characterization of *streptomyces*

Marine *streptomyces MW09-1* was isolated from Bay of Bengal water sample, athirampattinam, Thanjavur, Tamilnadu, India. Czapek Dox Agar used for isolation, consisted of (1g/l sea water) sodium nitrate 3g, dipotassium hydrogen phosphate 1g, Magnesium Sulphate 0.5, Potassium Chloride 0.5g, Ferrous

Sulphate 0.01g, Sucrose 30g, Agar Agar 15g, and pH 7, Temperature 25 °C. Microscopic and morphological Examinations were noted with respect to type of cells, aerial mycelium colour, nature of colony and reverse side plate colour.

2.2. 16sRNA analysis of Actinobacteria

16S rRNA gene sequences of isolates were compared to the non-redundant sequences database (GenBank) using the BLASTn program in the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Multiple sequence alignment was performed for homologous sequences and a phylogenetic tree was constructed using the neighbor joining method [6].

2.3. Isolation of Uropathogenic *E. Coli*

Uropathoenic *E. Coli* colonies were isolated from different age group of people. The isolated colonies are identified by microscopic, macroscopic and biochemical examination. The virulent factors are responsible for the pathogenicity of bacteria. Slime activity was performed by preparation Brain heart infusion agar plates with 0.8 gL^{-1} Congo red indicator. *E. coli* isolates were placed on the surface of the medium and the plates were incubated at 37 °C for 24 hour. Bright orange or red colonies were considered as positive. Another virulent factor Beta lactamase production was assayed using the method of Lateef. Broth culture of the test organism was spot inoculated on to Mueller-Hinton agar containing penicillin and 1% starch then incubated overnight at 37 °C. The presence of clear colourless zones around the bacterial growth indicates Beta lactamase production.

2.4. Antimicrobial activity

All isolates were subjected into antibiotic stability test according to methods suggested by Roy *et al., and* Bauer *et al.,* [7, 8]. The susceptibility of isolates of *E. coli* to antimicrobial agents was examined by a disc diffusion assay. Petri plates containing 20 ml of Mueller Hinton agar were seeded with 4 hours old fresh culture of clinical isolates and referral strains. By making use of template drawn discs were dispensed on the solidified Mueller Hinton agar with test organisms. This was incubated at 37 °C for 24 hours in an incubator (Rands SBC). The test was performed in triplicates. The zone of inhibition was measured by making use of Antibiotic zone scale (Hi - media). The resistance patterns were interpretive as per CDC recommendations.

2.5. Molecular characterization

Molecular methods are useful markers for the complete characterization of uro pathogenic *E. coli* strains [9, 10].

2.6. Molecular characterization

2.6.1. Amplification of Eae gene (Frans, 2013)

Amplification of *Eae* gene was carried out by using the reaction mixture consists of 2 µl of template DNA, 1 µl of 300 nM of each primer, 10 µl 2 X PCR master mixes (Promega, USA) and make up to 20 µl with molecular grade water. Amplification was performed in a Bangalore Genei thermo cycler. After initial denaturation at 95 °C for 1 min, the samples were subjected to 25 cycles of denaturation at 94 °C for 1min, annealing at 56 °C for 1 min. and extension at 72 °C for 10 min. A final extension was performed at 72 °C for 10 min. PCR products were examined by 1% (w/v) agarose gel electrophoresis in Tris Borate EDTA buffer (pH 8.2).

2.6.2. Multiplex PCR for the Identification of Multidrug Resistance Isolates

All the available partial and full-length gene sequences of resistance gene were determined according to protocol [11] with some modification. The primers were obtained from Sigma, India and used in the PCR comprised Primer.

2.7. 16sRNA analysis

2.7.1. Isolation of DNA from E.coli by amplification of 16srRNA gene sequence [12]

Amplification of 16sRNA gene sequence is done by centrifugation of *E. coli*. The culture was centrifuged at 8000 rpm for 5 minutes. The pellet was suspended with 200µl of 1X TE buffer and 100µl of 10% SDS and mixed by vortexing. The tubes were kept in water bath at 60 °C for 20 minutes and 300µl of Phenol: Chloroform: Isoamyl alcohol mixture (24:25:1) was added to extract the DNA and mixed completely by overtaxing. The tubes were then centrifuged at 10000 rpm for 10 minutes to separate the phases. The aqueous phase containing the DNA was carefully removed and transferred to new tubes. DNA solutions were stored at 4 °C for further work.

2.7.2. Methodology for Automated DNA sequencing

The following universal primers were used for the amplification of 16srRNA gene: F 5'AGA GTT TGA

TCC TGG CTC AG'3 and R 5'ACG GCT ACC TTG TTA CGA CTT'3. The cycle sequencing reaction was performed using Big Dye terminator V3.1 cycle sequencing Kit containing AmpliTac DNA polymerase (from Applied Bio systems, P/N: 4337457).

2.7.3. Data Analysis

16S rRNA gene sequences of isolates were compared to the non-redundant sequences database (GenBank) using the BLASTn program in the National Centre for Biotechnology Information (NCBI) website (http://blast.ncbi.nlm.nih.gov/Blast.cgi.). Multiple sequence alignment was performed for homologous sequences and a phylogenetic tree was constructed using the neighbor joining method [13].

2.8. GC-MS

GC-MS was done using the database of National institute Standard and Technology (NIST) having more than 62,000 patterns. The mass spectrum of the unknown component was compared with the spectrum of the known components stored in the NIST library. The name, molecular weight and structure of the components of the test materials were ascertained.

2.9. HPLC

HPLC analyses were carried out on an Agilent 1100 series HPLC, equipped with a quaternary pumping system, an in-line vacuum degasser, a variable wavelength diode array UV-visible absorbance detector, a 20-121 injection loop, and a personal computer with HP Chemstation software.

2.10. NMR

The purified fractions were analyzed by NMR spectroscopy for their identification and structure elucidation. The data obtained was compared with the reference data present in databases like Dictionary of Natural Products (DNP), *Anti Base* (a database of the microbial metabolites) and chemical abstracts.

2.11. Drug docking

2.11.1. Potential Target Structure

The 3D structures of intimin outer membrane protein from EHEC strain was accessed at RCSB server. The atomic co-ordinate file of intimin structure was obtained from the PDB (Berman *et al.,* 2000) [14].

2.11.2. Prediction of binding site

To determine the binding affinities between Diethyl phthalate, Ciproflaxcin and Intimin protein expressed by EAE gene of EHEC, the amino acids in the binding site of the Intimin outer membrane protein was predicted by submitting the structure to DoGSiteScorer: Active Site Prediction and Analysis Server [15].

2.11.3. Ligand generation

The 2D structures of Diethyl phthalate (CID: 6781) and Ciproflaxcin (CID: 2764) were retrieved from Pub chem. Database (Bolton *et al.,*) and minimized by using PyRx prior to the use for docking studies.

2.11.4. Flexible docking

The 3D structures of the Diethyl phthalate (CID: 6781) and Ciprofloxacin (CID: 2764) were docked with the binding sites of Intimin outer membrane protein of EHEC by using FlexX (Rarey et al, 1996) with following parameters i) default general docking information's, ii) base placement using triangle matching, iii) scoring of full score contribution and threshold of 0, 30 and No score contribution and threshold of 0,70. iv) Chemical parameters of clash handling values for protein ligand clashes with maximum allowed overlap volume of 2.9 A^{03} and intra-ligand clashes with clash factor of 0.6 and considering the hydrogen in internal clash tests. v) Default docking details values of 200 for both the maximum number of solutions per iteration and maximum number of solutions per fragmentation.

2.11.5. Prediction of ligand- receptor interactions

The interactions between the Diethyl phthalate (CID: 6781) and Ciproflaxcin (CID: 2764) and Eae expressed Intimin outer membrane protein from EHEC as docked complex were analyzed by the pose-view of LeadIT [16].

3. RESULTS AND DISCUSSION

Streptomyces sp., was isolated from marine water was found to produce active Phthalate for treating UTI bacterial infection. Antimicrobial activity, molecular Characterization and drug docking were determined against the test pathogens. Phthalate as the major active and non-toxic compound was separated and structurally suggested using GC-MS and NMR analysis.

Good fellow and Mukesh S *et al.,* [17, 18] reported that Biochemical characteristics of the *Streptomyces* are used for identification. Various biochemical tests were carried out to differentiate potential antimicrobial compound producing actinobacteria. When considering

Streptomyces MW09-1, it is a gram positive and non motile bacterium. It is nitrate negative and usease positive isolate. It also showed positive result for catalase and oxidase. They are also able to hydrolyse starch and casein, not gelatin (Table 1)

This sequence was submitted for genBank query using BLAST screening. Results of molecular characterization revealed that the strain MW09-1 showed 90% sequencing similarity with *Streptomyces champavatii*. Table 2 represented the 16S rRNA gene sequence analysis of MW09-1 strain. In general, information obtained using molecular techniques is very useful in that it provides researchers with a powerful and independent data set in which hypotheses generated from other data, such as morphology and physiology, can be tested [19]. Finally effective antagonistic isolate was identified as *Streptomyces sp*. This was subjected for antagonistic study using MDR-UPEC.

Table 1: Characteristic features of Stretomyces MW09-1 isolates

S.NO	Characters	Stretomyces
1	Areal mycelium	White
2	Substrate mycelium	White
3	Pigment	no
4	Spore chain morphology	retinoculiaperti
5	Gram staining	+ve
6	Nitrate	-ve
7	Urease	+ve
8	starch	+ve
9	casein	+ve
10	catalase	+ve
11	oxidase	+ve

Table 2: 16S rRNA Gene Sequence Analysis

S. No	Description	% Identity	Accession
1	*Streptomyces champavatii* strain KV2 16S ribosomal RNA gene, partial sequence	90%	KF454869.1
2	*Streptomyces champavatii* strain iafA 16S ribosomal RNA gene, partial sequence	90%	JN652251.1
3	*Streptomyces limosus* strain Act64 16S ribosomal RNA gene, partial sequence	89%	KT996112.1
4	*Streptomyces sp.* Act60 16S ribosomal RNA gene, partial sequence	89%	KT619159.1
5	*Streptomyces sp.* I07A-01484 16S ribosomal RNA gene, partial sequence	89%	GU550569.1
6	Uncultured bacterium clone 93LB-10 16S ribosomal RNA gene, partial sequence	88%	JQ272706.1

3.1. Assessment of Virulent factors.

In the present study, *Escherichia coli* topped the list of organisms causing UTI and proved itself as a major causative agent of UTI. The same was reported by by different workers from various parts of the world. Arasu, A.V *et al.,* [20] stated that *E. coli* was a causative agent in 78% UTI. Similarly Chen *et al.,* [21] in Taiwan reported *E. coli* as a common pathogen.

E.coli Strains fm different age group used for virulent factor assessment and other molecular studies were selected based on their resistance pattern. Most antibiotic resistance strains were subjected for molecular profile assessment and virulent assessment. Biofilm and β lactamase production ability is the major virulence determinant of uropathogens. Among the 9 isolates 8 isolates possess ESBL ability (Except E72). Biofilm formation is one of the major virulence factors of urinary pathogen. Six strains possess biofilm producing ability.

Strains E52, E53 and E72 were not able to produce biofilm during study period (Table 3). Thought our results were in line with the report given by Magarvey NA *et al.,* [22], who stated that almost 60% of isolates produced two or three virulence factors; only 3.8% produced none of the virulence factors.

Table 3: Assessment of Virulence factors

S. No	Organism ID	Biofilm	β Lactamase
1	E44	+	+
2	E51	+	+
3	E52	-	+
4	E53	-	+
5	E54	+	+
6	E55	+	+
7	E56	+	+
8	E71	+	+
9	E72	-	-

3.2. Plasmid mediated antibiotic resistance isolates by PCR

Antibiotic resistance of bacteria could be due to specific genes like TEM, SHV, OXA, and CTX_M. These genes were found in R plasmid of a particular plasmid. Six of the UPEC strains were subjected for the amplification of a resistance gene. Results of amplification of a resistance gene revealed that TEM gene was found in all the isolates. CtxM gene was found in E44 and E51 isolates. SHV hgene is found in three isolates (E44, E51 and E55). OXA gene was found in only one strain E51 (Table 4). (Plate I) clearly illustrated the presence of antibiotic resistance genes in uropathogenic isolates, whose evidence is based on the molecular weight markers and the base pair weight of the particular gene. Berman HM *et al.,* [23] also reported that TEM gene was detected in 28 % of isolates, SHV gene in 74 % and CTX-M gene was detected in only 2.5% isolates.

Table 4: Nature of virulence genes among UPEC isolates

S. No	Isolates	Virulence genes			
		TEM	SHV	OXA	CtxM
1	E44	+	+	-	+
2	E51	+	+	+	+
3	E54	+	-	-	-
4	E55	+	+	-	-
5	E56	+	-	-	-
6	E72	+	-	-	-

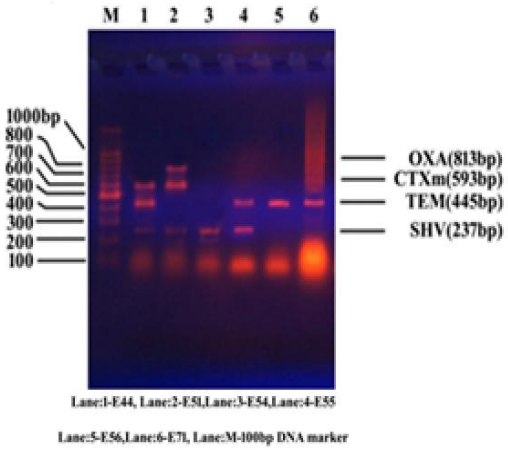

Plate-I: Assessment of ESBL genes in UPEC Isolates

3.3. Assessment of Eae gene

Categories of *E. coli* that differs in their virulence factors contain *eae* gene encoding for intimin as part of Pathogencity Island EPEC and EHEC [24]. Based on the reference**,** all the 9 pure UPEC were subjected for the assessment of *eae* gene. Assessment of *eae* gene revealed the presence of *eae* gene in six strains. (Plate II) revealed 397 bp gene fragments, which could be an *initimin* gene responsible for pathogenicity of the isolates. E54, E56 and E71 didn't show any eae gene.

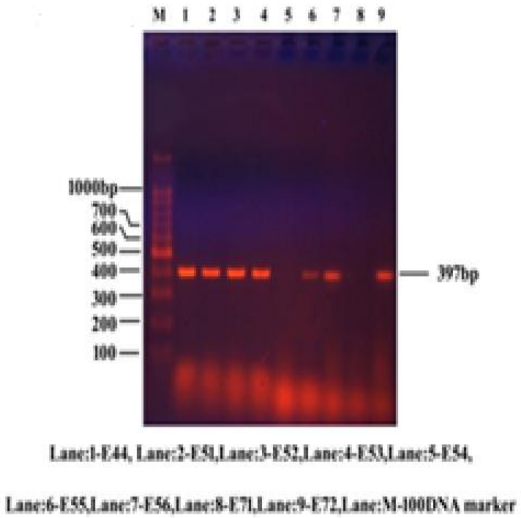

Plate II: Amplification of EAE Gene

3.4. Screening of antimicrobial activity

Out of thirteen selected and identified actinobacteria, *Streptomyces* sp., MW09-1 showed significant antimicrobial activity against multidrug resistant UTI pathogens. Five pathogenic strains isolated from cases of UTI infection were used as a test organism for antagonistic study. Among the actinobacteria tested, *Streptomyces* sp., MW09-1 strain produced the best activity against all the test organisms (Table-5) at 100µl /disc concentrations against MDR urinary isolates. The results indicated that all the actinobacterial strains showed good antibacterial activity. *Streptomyces* sp., MW09-1 (RW2-3) strain produced a 16.6±2.08 mm zone of inhibition against E51 and E44 strains of UPEC. Best antimicrobial activity was exhibited by MW09-1 strains. This strain was considered as a *Streptomyces* sp. (Table 5). In this present study biological screening was done against *E.coli* UTI pathogen, similar kind of antagonistic activity also reported by different authors from India but they used different pathogens [25-27].

Overall GC-MS analysis revealed the presence of molybdenum complex, spiro compounds, iodo compounds, chlorocompounds, Diethyl phthalate, butyl phthalate, phthalic acid mono ester, phthalic acid diester, acetyl benzoic acid were the major compounds present in fermented extracts of *Streptomycis* MW09-1. Similar compounds were also noted in plant extracts. In

a similar study, the natural occurrence of 1, 2-Benzenedicarboxylic acid bis (2-ethylhexyl) phthalate has been isolated from a marine alga, *Sargassum weightii*. It is a plasticizing agent. It was also found to have antibacterial effect on a number of bacteria [28].

Table 5: Screening of *Streptomyces* sp MW09-1(RW2-3) for its antagonistic activity

S. No	Organism ID	Zone of inhibition in (mm)
1	E-51	16.6±2.08
2	E-44	16.6±2.08
3	E-71	12.3±0.57
4	E-52	13.0 ±2.0
5	E- 53	12.3±0.57

In this study, HPLC analysis was used for the determination of diethyl phthalate esters (DEP) present in the sample with the help of purified standard diethyl phthalate. Further this phthalate ester was completely separated from the other components of the sample by HPLC and was quantified within the range 0.001 mg/ml to 0.3 mg/ml which was also conformed using HPLC calibration curves obtained using different concentration as per standard linear concentration of DEP used. Similarly, Natural production of phthalate from *S. bangladeshiensis* showed antimicrobial activity against some pathogenic Gram positive bacteria [29].

Further, the purified sample was characterized for functional group analysis using ^{1}H NMR. The characteristic intense chemical shift indicated in the region of 7.612ppm represents the molecular structure of aromatic ring, 2.645ppm and 2.951ppm indicating the presence of ethyl group with oxygen and the peak observed at 2.112ppm corresponds to the presence carbonyl group (C=O) in the compound. The chemical shifts present in the region 3.631ppm depicted the presence of dissolving solvent DMSO. The above observation made with the molecular structure of the purified compound proves that it could be a diethyl phthalate. Plate II illustrates the NMR pattern of *Streptomycis* MW09-1 culture filtrate. Results indicated that diethyl phthalate is a major compound along with molybdenum complex. It was also supported by Anusha *et al.,* [30].

3.5. Docking studies

In general the *Escherichia coli* are characterized by their ability to cause attaching-and-effacing (A/E) lesions in the gut mucosa of human and animal hosts that leads diarrhoea. The main mechanism of *Escherichia coli* pathogenesis is the destruction of the gastric microvillus brush border through restructuring of the underlying cytoskeleton by signal transduction between bacterial and host cells, intimate adherence of strains to the intestinal epithelium, pedestal formation and aggregation of polymerized action at the sites of bacterial attachment. The adherence of bacteria to the enterocytes is mediated by intimin, an outer membrane protein encoded by the eae (*E. coli* attachment effacement gene Frans I *et al.,*) [31] thus considering this Intimin protein as a potential drug target. This study assessed binding efficiency of Diethyl phthalate (DEP) from source name and ciprofloxacin as a standard antibiotic.

The activity of the Diethyl phthalate extracted from source is determined by docking within the predicted binding sites of Intimin protein expressed by eae gene of UPEC. It is observed that Diethyl phthalate docked with Intimin protein with a dock score of -18.9346 kJ/mol. This docking interactions is favoured by the formation H-bond between amide groups (-NH) of Leu230, Arg201, Arg221 and the keto groups (=O) of Diethyl phthalate. Further the interaction is supported by Gln232, Lys94, Leu230, arg201 and Arg229 with hydrophobic interactions (PlateIII). It is observed that the Arg 201 favoured both H-bond formation and hydrophobic interactions, which envisages that Arg 201 plays a crucial role in binding of Diethyl phthalate with Intimin protein of UPEC.

The major component in all the isolate was phthalate reported Dibutyl phthalate, isobutyl phthalate were active against partially four selected immortal cell lines. In a similar study the natural occurrence of 1, 2-Benzene dicarboxylic acid bis (2-ethylhexyl) phthalate has been isolated from a marine alga, *Sargassum weightii*, and apart from its plasticizing ability it was also found to have antibacterial effect on a number of bacteria. Bis (ethyl hexyl) phthalate reported from *Streptomyces bangladeshiensis* show antimicrobial activity against gram positive bacteria and some pathogenic fungi.

In line with this, the binding efficiency of Ciproflaxcin was also determined by docking with the same predicted pocket of Intimin protein expressed by eae gene of UPEC. It is observed that the ciprofloxacin binds more effectively when compared to that of Diethyl phthalate as it exhibited the dock score of -25.3388 kJ/mol.

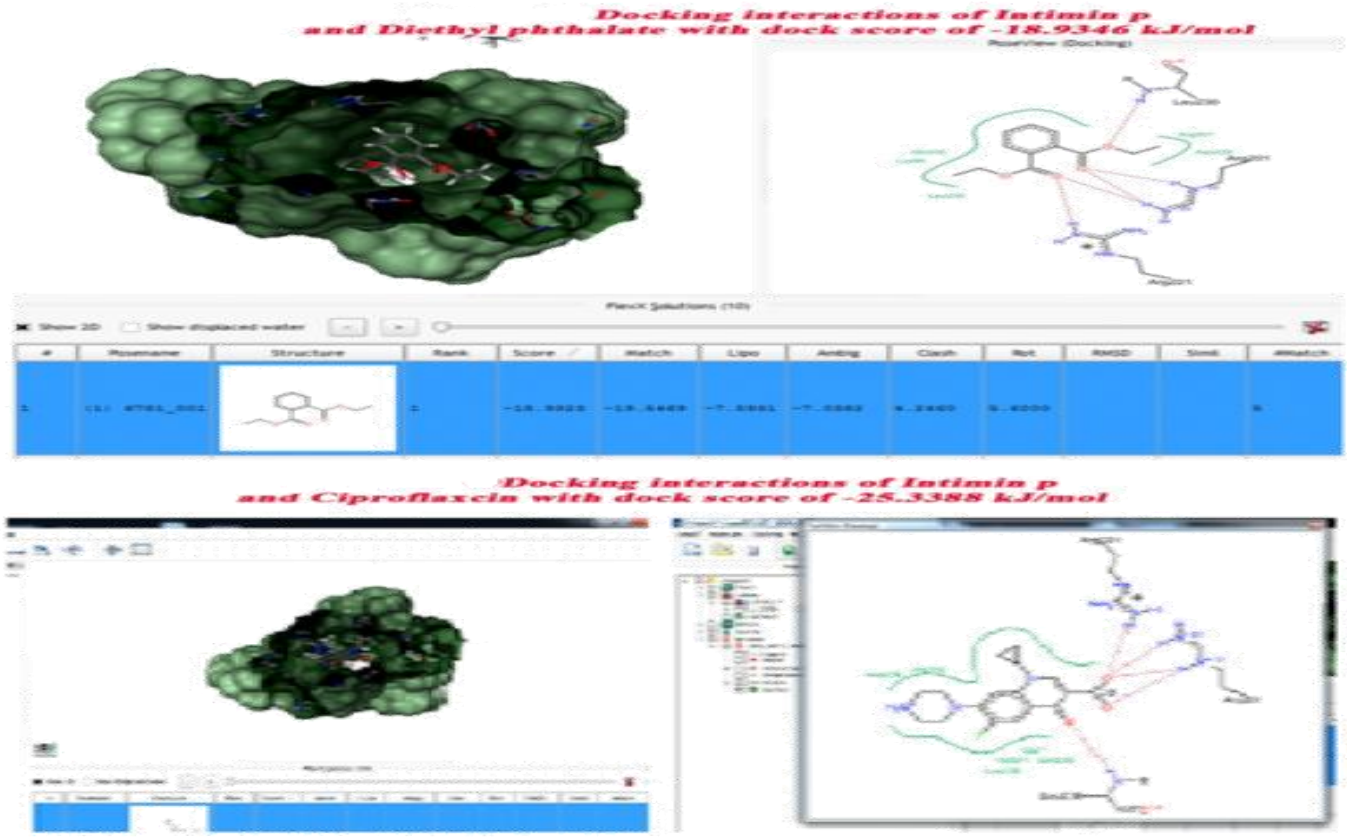

Plate III: Drug Docking

This interaction is favored by H- bonds between amide groups (-NH) of Leu 230, Arg201, Arg221 and the keto groups (=O) of ciprofloxacin and Hydrophobic interactions by Arg233, Asp229, Glu7, Gln232, Val231 and Leu230 (Plate III). It is observed that Leu230 plays a crucial role in binding of ciprofloxacin by forming both the H bond interactions and hydrophobic interactions.

Interestingly it is observed that both the Diethyl phthalate and Ciproflaxcin docked in the predicted active site of intimin protein is favoured by the H-bond formation by the same amino acids Leu230, arg201, arg221 and hydrophobic interactions by Gln232, Leu230, Arg229 which revealed that both the compounds exhibits the drug ability to inhibit the virulence caused by intimin protein expressed by eae gene of UPEC.

Further this study revealed the role of Arginine and Leucine for both H-bond interactions and hydrophobic interactions might be considered as crucial amino acids while designing novel inhibitory compounds that can efficiently inhibit the Intimin outer membrane protein expressed by eae gene of UPEC. From this result, we may conclude that this compound is a natural compound with minimum side effects unlike synthetic or semi synthetic antibiotics.

4. CONCLUSION

This also confirms antimicrobial nature of *Streptomyces* MW09-1 extracts against MDR, bio film producing virulent UPEC isolates of UTI. To implement the illustrations of this study for the benefit of human beings, it is necessary to carryout clinical trials. It is recommended that, the *Streptomyces* isolated from sea water could be acted as antagonistic organisms against MDR-UPEC. Further studies are needed to confirm pharmacological values of actinobacterial compounds.

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