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Original Article

SYNTHESIS, EVALUATION AND DOCKING STUDIES OF NOVEL FORMAZAN DERIVATIVES AS AN ENOYL-ACP REDUCTASE INHIBITORS

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ABSTRACT

Objective: To synthesize, evaluate and performing the docking studies of novel formazan derivatives as enoyl-ACP reductase inhibitors.

Materials: In the present investigation, a series of formazans (Ia-d) were synthesized by stirring aryl diazonium salts solution with Schiff's base at 0-5˚C for 2 h. The intermediate azomethine (Schiff base) itself was synthesized by condensation of para aminobenzoic acid with dimethylamino benzaldehyde in presence of a glacial acetic acid as a catalyst. The antimicrobial activity was done for these synthesized compounds by cup plate method. Moreover, the antimicrobial activity was further confirmed by its molecular docking approach study by using Molecular Operating Environment (MOE) 2009.10 software.

Results: In the present study all the synthesized compounds (Ia-Id) showed the enhanced zone of inhibition against S. aureus, B. subtilis, E. coli and S. typhi (5±0.12 to 12±0.45) whereas, the antifungal activity against A. niger and C. albicans were showed the zone of inhibition in the range of 9±0.51 to 12±0.43 when compared to that of the standard drug.

Further the docking study reveals that, only three of the formazan compounds under observation (Ia, Ib and Ic) have higher binding affinity with the receptors enzymes enoyl-ACP reductase, which is in the narrow range of binding energy for the protein PDB: 1C14 is-24.4598 to-23.9377 kcal/mol, which shows the further confirmation of these formazan compounds as better microbial inhibitor.

Conclusion: Therefore our present report shows that formazans could be the potential drug candidate that inhibits the microbial activity by interacting and inhibiting the enoyl-ACP reductase enzyme which is confirmed by its both *in vitro* antimicrobial study and as well as from its docking study.

Keywords: Formazans, Schiff base, Enoyl-ACP Reductase, Docking, MOE

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INTRODUCTION

Large use of antibiotics against pathogenic microorganism has a lot of toxic and side effects. Owing to this, a potential research in search of alternative options is required to combat with the resistance of bacteria [1]. There are numerous antibiotics have been produced, but the clinical efficacy of these existing antibiotics is being threatened by the emergence of multidrug-resistant pathogens [2]. In the search for safer and more potent therapeutic agents, a popular approach is synthesis and evolution of biologically active compounds. Azomethine derivatives have been prominent research subject because of their pharmacological characteristics and complexometric behaviour. Azomethine derivatives play a pivotal role in displaying various biological activities viz. anticancer, antitubercular, anti-HIV, antioxidant and antimicrobial. Azomethines are the important intermediate for the synthesis of derivatives such as pyrazolines, isoxazolines, azetidinones, thiazolidinones and formazans.

Formazans compounds have the general structure with an azohydrazon arrangement of functional groups. Independently Bamberger and Von Pechman synthesized the first formazan afterwards there have been numerous formazans synthesized and investigated. Formazans are coloured compounds ranging from cherry red to a deep purplish black and contain atoms of (-N=N-C=N-NH-) because of their $\pi \rightarrow \pi^*$ transitions of π -electrons. Formazans are generally solids of relatively low melting point compound with huge the size of its structure [3]. Formazans have been studied widely owing to their ready ease of access, assorted chemical reactivity and its biological activities [4, 5]. Formazans have attracted attention because of their diverse medicinal applications such as antimicrobial [6-15], (including antiviral, antifungal, antibacterial), antifertility [16], analgesic and anti-inflammatory [17-20], antitubercular [21, 22], anticonvulsant [23, 24], antiparkinsonian [25, 26], anticancer, anti HIV [27-29], antiproliferative [30, 31] and antihyperglycemic [32] activities.

Owing to the universal impact of these devastating diseases, there is an urgent need for the development of new derivatives with promising antimicrobial activities. Several different approaches such as targeting bacterial virulence, high-throughput screening (HTS), structure-based drug discovery (SBDD), chemical modifications of the known drugs and combinatorial chemistry have been discovered to search novel biologically important lead [33-35]. Apart from this, now a day, the new concept of computer-aided drug design (CADD) and docking has revolutionized the entire drug discovery and development program.

Formazan derivatives were known to possess various pharmacological activities. Hence it was decided to synthesize new derivatives of formazan by coupling reaction. The goal of the present study is to synthesis compounds with greater efficacy, safety and less toxic for the use of medicinal purpose. In the light of these observations and diverse literature survey, our attention was drawn towards to synthesis and study of few newer formazan derivatives and was evaluated for antimicrobial activities.

In support with this *in vitro* antimicrobial activity result and by taking into consideration of NADH-dependent enoyl-Acyl Carrier Protein reductase (enoyl-ACP reductase) as the target receptor of the antimicrobial activity [36], it was focused to perform the molecular docking studies and screening for supportive information between *in silico* studies with the *in vitro* results. Docking studies

with newly synthesized compounds were performed to determine the best *in silico* conformation. Due to this, an attempt was made in the present research to design, synthesis and docking studies of novel formazans derivatives as an anti-*E. Coli* mediator having enoyl-ACP reductase inhibitor.

MATERIALS AND METHODS

The chemicals used of AR and LR grade and were obtained from Merck, Hi-Media and Sigma-Aldrich, SD Fine chem., Mumbai. All the chemicals were used as received without further purification.

Experimental section

Melting points were determined in open capillaries on a Thomas Hoover apparatus and are uncorrected. The synthesized compounds were characterized by the following methods. IR spectra of synthesized compounds were recorded on a Schimadzu Fourier Transform Infrared Spectrophotometer in the range of 4000 cm^{-1}– 400 cm^{-1} using KBr pellet technique. Proton NMR Spectra were recorded using BRUKER Advance 500MHz NMR Spectrometer using the solvent deuterated chloroform. Chemical shifts were recorded in parts per million and Trimethylsilane as an internal standard.

General procedure for synthesis of new formazan derivatives

Synthesis of new formazans involved two steps. In the first step, azomethine was synthesized by the reaction of a para-aminobenzoic acid and dimethylamino benzaldehyde in presence of an acidic catalyst glacial acetic acid. In second step formazans were synthesized from azomethine (Schiff base) by reaction with various aryl diazonium salt at 0-5 °C in the presence of pyridine.

Synthetic procedure

Step 1: Synthesis of 4-{[4-(dimethylamino) benzylidene] amino} benzoic acid (I)

0.02 mol of para-aminobenzoic acid and 0.02 mol of para dimethylamino benzaldehyde were dissolved in 30 ml of ethanol. Then few drops of glacial acetic acid were added to it and the contents were refluxed for 1h. The reaction mixture was cooled and poured into water. The yellow colour solid product was separated. This was filtered, washed with water, dried and recrystallized from ethanol. Formation and purity of products were checked by thin layer chromatography using chloroform, methanol as mobile phase and silica gel G as the stationary phase and the spots were visualized by iodine vapour.

Step 2: Synthesis of Aryl diazenyl (dimethyl aminophenyl) methylidene aminobenzoic acid derivatives [I(a-d)]

0.006 mol of different aromatic amines dissolved in 15 ml of 2 M hydrochloric acid. The contents were cooled to 0-5 °C in an ice bath. The cold solution of sodium nitrite (0.012 mol in 10 ml water) was slowly added to the amine solution with stirring and maintain the temperature at about 0-5 °C.

0.004 mol of product (I) obtained in step 1 was dissolved in 10 ml of pyridine and the mixture was cooled in an ice bath at 0-5 °C. To this mixture, the cold diazotized solution was added dropwise with stirring and stirring was continued for 2h. The reaction mixture kept in an ice bath for further 3h. The coloured solid product was separated and it was filtered, washed with a minimum quantity of water, dried and recrystallized from ethanol. The overall synthetic scheme represented as fig. 1.

Antimicrobial activity

Antifungal activity and antibacterial activity

The newly synthesized compounds were subjected for antifungal activity [10, 14] against fungi such as *A. niger* (ATCC 16404) and *C. albicans* (ATCC 18804), and antibacterial activity [10, 14] against gram-positive bacteria such as *S. aureus* (MTCC 1133) and *B. subtilis* (MTCC 7443) and gram-negative bacteria such as *E. coli* (MTCC 1692) and *S. typhi* (ATCC 19430). All the bacterial and fungal strains were purchased from VNS enviro biotech pvt ltd., Chennai, Tamilnadu. The activity was done by cup plate agar diffusion method.

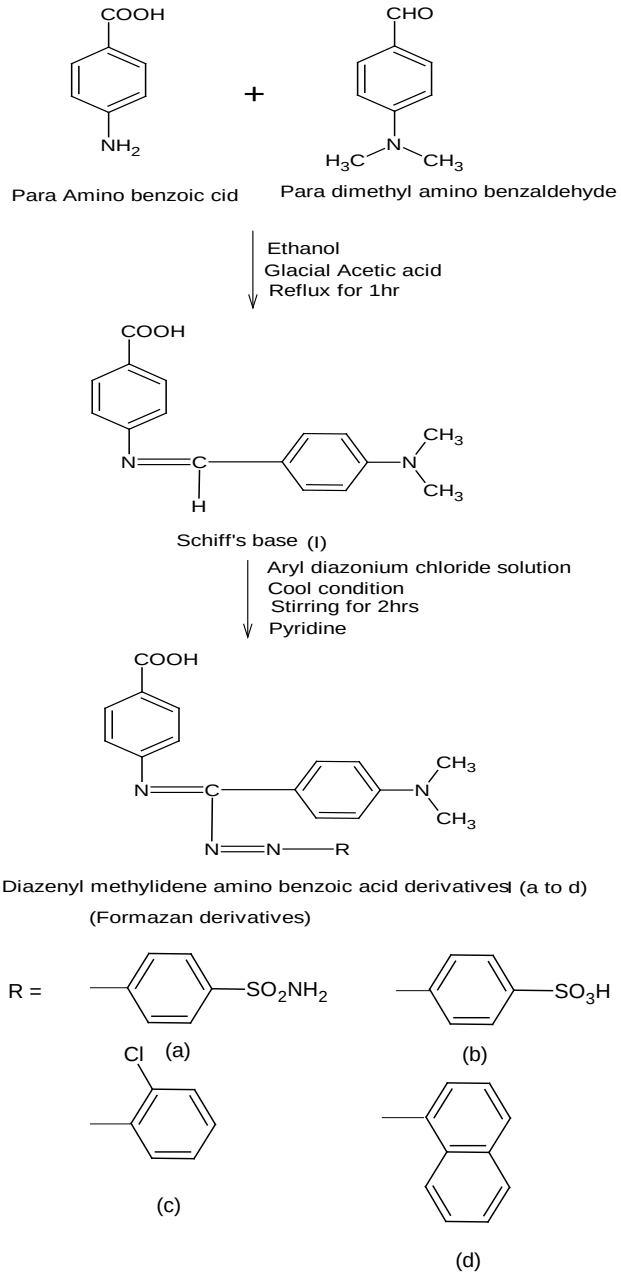

Fig. 1: Synthetic scheme

Molecular docking studies

Preparation of ligand files

The ligand files for the molecular docking studies were prepared in the Molecular Operating Environment (MOE, 2009.10) by Chemical Computing Group (CCG). The molecular geometries were drawn, correct 3D structures were ensured and were followed by energy optimization at a standard MMFF94 force field level, with a 0.0001 kcal/mol energy gradient convergence criterion [37]. The module builder of MOE program was used for this purpose and after building the molecule it was saved as a molecular database (.mdb) file for further studies.

Preparation of receptor

The first step of the protein preparation involved removal of water and hetero molecule by using sequence editor (SEQ) window which is default in MOE programme. Further, the energy was minimized followed by active site prediction. The crystal 3D structure of enzymes enoyl-ACP reductase (PDB: 1C14), was obtained from

Protein Data Bank (http://www.rcsb.org/pdb). The pdb file was imported to MOE suite where receptor preparation module was used to prepare the protein. All the bound water molecules were removed from the complex. Both polar and non-polar hydrogens were added and 3D structure was corrected. The 3-D protonated structure was energy minimized. Since the protein was devoid of associated ligand, so the pocket was identified using the active site finder module of the MOE. In order to visualize the binding pocket, alpha spheres were created followed by the generation of dummy atoms on the centres of these spheres. The pockets were found to be deep small gorges lined with the key residues including both hydrophobic and hydrophilic amino acids.

Docking methodology

The optimized ligands were docked with the enzymes enoyl-ACP reductase (PDB: 1C14) using the MOE-Dock program. For docking simulations, the placement was set as triangular matcher, rescoring was set as London dG, the number of retaining was set as 10 and the refinement was set as forcefield on MOE docking and was utilized to generate 10 poses of each compound. As a result of the docking run, the .mdb output files were created with scoring and multiple conformations of each compound. All the docked conformations were analyzed and the best-scored pose for each compound was selected for further interaction studies.

RESULTS AND DISCUSSION

The target compounds were synthesized by coupling reaction of Schiff's base and various aryl diazonium salts. The synthesized compounds were obtained in moderate yield. The percentage yield and melting point of the synthesized compounds were recorded and presented uncorrected. Thin layer chromatography was performed for the synthesized compounds. The only single spot was obtained on silica gel G plate as a stationary phase and chloroform, ethanol as a solvent system. This showed that the compounds were obtained in a pure state. IR and ^{1}H NMR Spectral data of synthesized compounds were given below.

Compound Ia: 4-({[4-sulphamoyl phenyl) diazenyl] [4-(dimethyl amino) phenyl] methylidene} amino) benzoic acid. Yield 67%. m. p. 164 ° C. IR γ (cm^{-1}) (KBr): 3306-primary sulphonamide–NH stretch, 3182-3076 (OH stretch-COOH), 3026-CH stretch of aromatic, 2999-2991-CH stretch of methyl, 1672-C=O-stretch of–COOH, 1606-C=N stretch, 1591and1521–C=C stretch of aromatic, 1425-N=N-stretch, 1178-=S=O-stretch. ^{1}H NMR δ ppm (TMS): 0.9-1.3–6H (CH_3), 3.3-3.8–2H (SO_2NH_2), 8-8.5–12 H.

Compound Ib: 4-({[4-sulphophenyl) diazenyl] [4-(dimethylamino) phenyl] methylidene} amino) benzoic acid. Yield 72%. m. p. 228 ° C. IR γ (cm^{-1}) (KBr): 3308-OH stretch of SO_3H, 3182-OH stretch of–COOH, 3030-CH stretch aromatic, a 2991-CH stretch of CH_3, 1670-C=O stretch of–COOH, 1606-C=N stretch, 1591 and a 1521-C=C stretch of aromatic, 1425-N=N stretch. ^{1}H NMR δ ppm (TMS): 0.9-1.4–6H (CH_3), 2.3–1H (SO_3H), 7.4-7.9–12H.

Compound Ic: 4-({[2-chlorophenyl) diazenyl] [4-(dimethyl amino) phenyl] methylidene} amino) benzoic acid. Yield 68%. m. p. 148 ° C. IR γ (cm^{-1}) (KBr): 3201-OH stretch COOH, 3082-CH stretch aromatic, 2995-CH stretch CH_3, 1718 and 1683-C=O stretch COOH, 1602,-C=N stretch 1502,-C=C stretch, 1442-N=N stretch. ^{1}H NMR δ ppm (TMS): 1.0-2.9–6H (CH_3), 6.7–6.9–4H (Ar H), 7-7.5–4H (ArH), 7.9-8.2–4H (ArH).

Compound Id: 4-({[(1-naphthyl) diazenyl] [4-(dimethylamino) phenyl] methylidene} amino) benzoic acid. Yield 56%. m. p. 189 ° C. IR γ (cm^{-1}) (KBr): 3113-OH stretch-COOH, 2987-CH stretch aromatic, 2949-CH stretch CH_3, 1681-C=O stretch–COOH, 1599-C=N stretch, 1548 and 1525-C=C-stretch aromatic, 1435-N=N-stretch. ^{1}H NMR δ ppm (TMS): 1.3–3H (CH_3), 3.2–3H (CH_3), 6.7–9.8–15 H (ArH).

Antimicrobial activity

Formazans have been reported to possess an attractive antimicrobial activity [6-15]. Further to confirm this, the zone of inhibition value of the all the synthesized compounds (Ia-Id) were showed good antifungal and antibacterial activity when compared to that of the standard drug. The activity may be due to the presence of electron withdrawing groups (-SO_2NH_2,-SO_3H,-Cl) in synthesized formazan compounds Ia–Id. The results were summarized in table 1 and 2 respectively.

Table 1: Antifungal activity (zone of inhibition in mm) of the synthesized compounds

Compound	*Aspergillus niger*				*Candida albicans*			
	Concentration (µg/ml)				Concentration (µg/ml)			
	500	800	1250	2000	500	800	1250	2000
Ia	-	-	9±0.51	10±0.35	-	-	7±0.31	-
Ib	-	-	12±0.43	9±0.31	-	-	6±0.33	7±0.26
Ic	-	-	10±0.35	9±0.41	-	-	12±0.54	11±0.40
Id	-	-	-	8±0.52	-	-	-	7±0.13
Standard (250µg/ml) (Griseofulvin)	28±0.46				24±0.22			

Values are mean of triplicate readings (mean±SD); (-) indicates no zone of inhibition

Table 2: Antibacterial activity (zone of inhibition in mm) of synthesized compounds

Compound	Gram positive bacteria				Gram negative bacteria			
	S. aureus		*B. subtilis*		*E. coli*		*S. typhi*	
	Concentration (µg/ml)				Concentration (µg/ml)			
	250	1250	250	1250	250	1250	250	1250
Ia	7±0.36	8±0.43	-	-	-	-	-	6±0.13
Ib	6±0.32	7±0.40	-	-	7±0.22	8±0.51	6±0.21	9±0.43
Ic	7±0.12	8±0.31	8±0.41	12±0.65	14±0.65	11±0.53	7±.26	12±0.45
Id	5±0.12	6±0.32	8±0.56	11±0.38	-	-	-	-
Standard (250µg/ml) (Gentamycin)	18±0.56		17±0.35		15±0.53		14±0.54	

Values are mean of triplicate readings (mean±SD); (-) indicates no zone of inhibition

Molecular docking study of the synthesized formazans

In the present study, the docking of novel formazan derivatives as an anti-*E. Coli* mediator having enoyl-ACP reductase inhibitor was performed. Enoyl-acyl carrier protein reductase is a fatty acid synthase II enzyme involved in the bacterial fatty acid biosynthetic pathway of the bacteria. This enzyme also involved in fatty acid elongation in the cell wall synthesis of bacteria [36].

From the docking result, it was observed that the docked conformations for each compound were analyzed and it was found that the most favourable docking poses with a maximum number of

interactions were those which were ranked the highest based on the minimal binding energy, which was computed as a negative value by the software. The most favourable docking poses of the 10 docked conformations for each compound were analyzed to further investigate the interactions of the docked conformations within the active sites. The tremendous number of interactions with active site residues coupled with favourable binding energy proclaim that these compounds may serve as an effective replacement for the antimicrobial agents. These ligands showed a proper binding pattern and anchored tightly inside the active site gorge (Site I) of the protein. The 2D ligand-protein interactions were visualized using the MOE ligand interaction program and recorded in table 3.

Table 3: Docking results for formazans derivatives with protein PDB: 1C14

Compound no	S	rmsd_refine	E_conf	E_place	E_score1	E_refine	No. of conf.
Ia	-24.4598	3.8591	-16.2233	-103.6424	-12.3635	-24.4598	10
Ib	-24.8136	3.0560	30.4684	-94.1171	-13.6685	-24.8136	10
Ic	-23.9377	1.6005	87.5645	-107.4476	-11.6685	-23.9377	10
Id	-	-	-	-	-	-	-

S-The final score, rmsd_refine-The root mean square deviation between the pose before refinement and the pose after refinement, E_conf-The energy of the conformer. E_place-Score from the placement stage, E_score1-Score from the rescoring stage(s), E_refine-Score from the refinement stage and No. of conf-number of conformations generated by ligand.

The best docking poses of all 4 selected compounds produce results in which Ia, Ib and Ic form a single cluster inside the active site cleft of the receptor as shown in fig. 2, 3 and 4. The compounds under observation have a higher binding affinity with the receptors, in the narrow range of binding energy for the protein 1C14 is-24.4598 to-23.9377 kcal/mol: London dG is-13.6685 to-11.6685 kcal/mol. Whereas, the compound Id was not shown any binding score with this protein. This was further confirmed by its *in vitro* antimicrobial activity data which is present in table 1 and table 2.

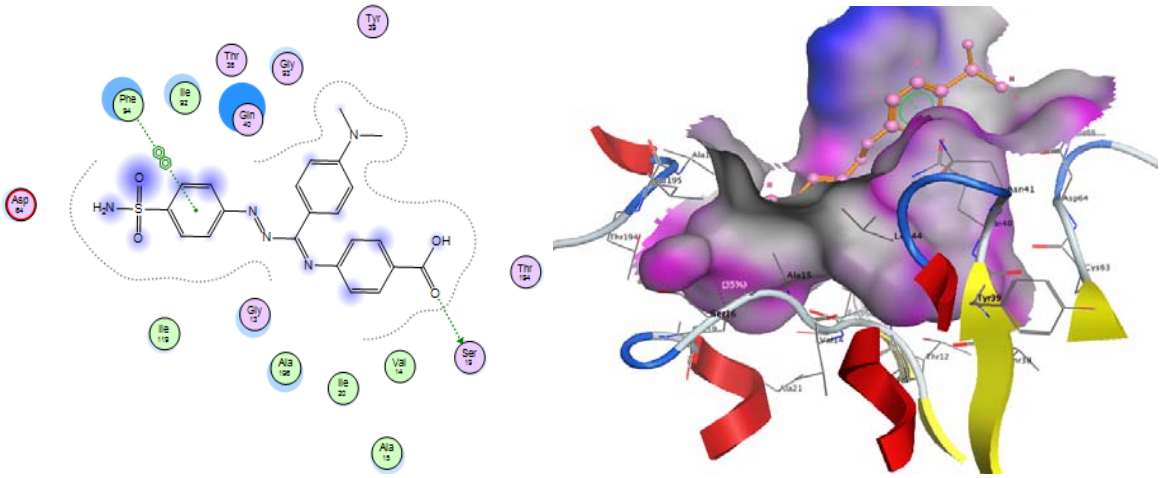

Fig. 2: Ligand receptor interaction and binding surface of compound Ia with 1C14

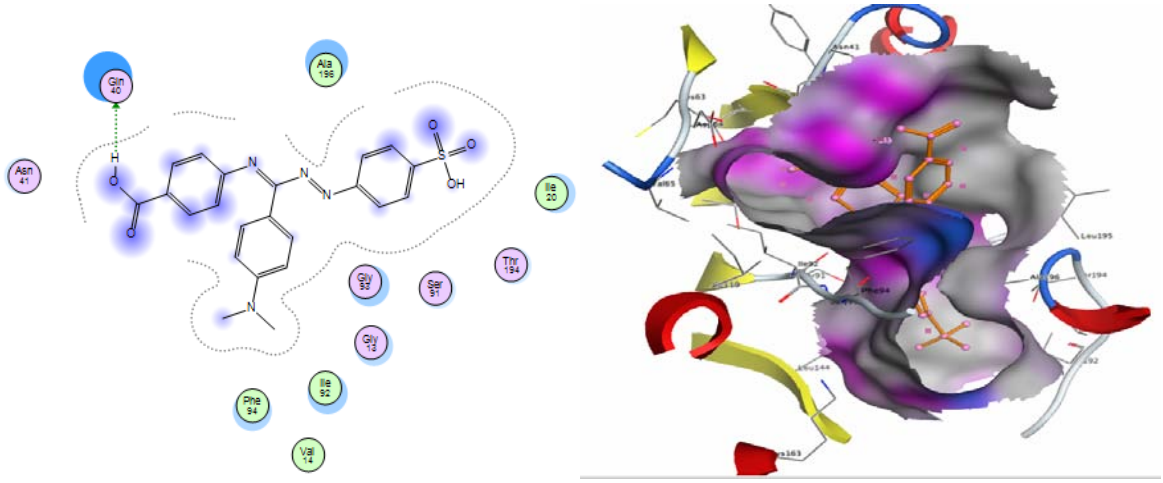

Fig. 3: Ligand-receptor interaction and binding surface of compound Ib with 1C14

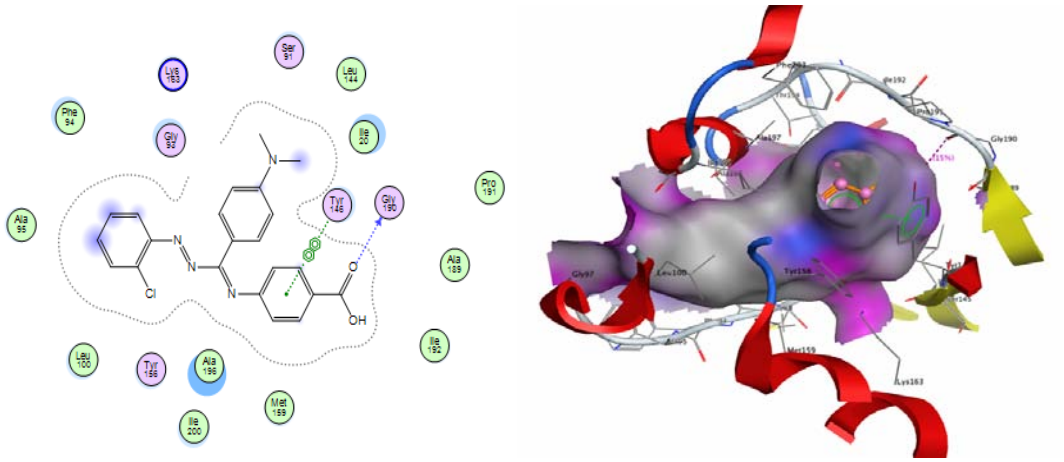

Fig. 4: Ligand receptor interaction and binding surface of compound Ic with 1C14

Docking analysis revealed that the compound Ia interacted with receptor through arene-arene interactions on Phe 94, and also there is a side chain acceptor on carbonyl group of acid moiety ser 16, while in Ib there is a side chain acceptor on carbonyl center of acid moiety at Gln 40, whereas compound Ic interacted with receptor Tyr 146 through arene-arene interactions and there is a backbone on carbonyl group of acid moiety at Gly 190, (fig. 2, 3 and 4). The number of conformations generated by compound Ia, Ib and Ic were 10 which indicated that flexibility is an important parameter for the ligand to docked deeply within the binding pocket of enoyl ACP reductase enzyme. The energy of confirmation for compound Ia is-16. 2233, Ib is 30.4684 and Ic is 87.5645, which indicate compound is active at this energy of confirmation. Further, a careful inspection of the binding pocket indicated that compounds Ia, Ib and Ic adopted a position in a hydrophobic cage surrounded by Tyr 39, Ala 15, Leu 195, Asp 64, Cys 63, Phe 94, Ser 16 and etc.

CONCLUSION

In the present study, the zone of inhibition value of the all the synthesized compounds (Ia-Id) was showed good antifungal and antibacterial activity when compared to that of the standard. The activity may be due to the presence of electron withdrawing groups (-SO_2NH_2,-SO_3H,-Cl) of synthesized compounds Ia–Id. Further, the docking study reveals that, the only three compounds under observation (Ia, Ib and Ic) have a higher binding affinity with the receptors enzymes enoyl-ACP reductase, in the narrow range of binding energy for the protein PDB: 1C14 is-24.4598 to-23.9377 kcal/mol. Whereas, the compound Id was not shown any binding score with this protein. This was further confirmed by its *in vitro* anti-microbial activity data.

AUTHORS CONTRIBUTIONS

T. Prabha-Who is fully contributed to preparing this whole manuscript to write up, followed by a plagiarism check if any, and to check the grammar by using the Grammarly online software. Moreover, she is the one who runs the docking analysis by using MOE 2009.10 software. C. Selvinthanuja-Who is actively, involved in the synthesis of compounds. T. Sivakumar-With his guidance, the author moved further to complete this work and also he guided to write the manuscript and finally the proofreading of this manuscript was done by him.

CONFLICTS OF INTERESTS

The authors have no conflicts of interests

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