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In Vitro Analysis of Antimicrobial Compounds from *Euphorbia milli*

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Abstract

Pharmaceutical and scientific communities are now looking at newer avenues of obtaining antimicrobial agents, and the focus has now been on natural sources. In this study, *Euphorbia milli* extract was characterised for antimicrobial activity. The antimicrobial effect was determined by calculating minimum inhibitory concentration against the pathogens *Pseudomonas aeruginosa*, *Escherichia coli, Salmonella typhimurium*, *Staphylococcus aureus, Streptococcus pneumonia, Klebsiella pneumoniae*, and *Shigella dysenteriae*. The active metabolites of the plant extract manifested a significant antibacterial effect. In silico analysis was carried out using iGemdock as the docking tool for docking Penicillin Binding proteins against the extracted active metabolites. The docking score Hexamethlyl-cyclotrisiloxane / Tolmetin emerged as the most effective active metabolite against all the pathogens except Shigella dysenteriae N-Hexadecanoic acid was the best choice.

Keywords: Bioactive metabolites, Extraction, bioactivity, Pathogens,

Introduction

COVID-19 has thrown the whole world upside down, scrapping for a viable cure and survival due to the lack of an effective curative agent. Co-morbidities and increasing resistance of human pathogens to traditionally adopted antibiotics have altered our perspective for searching for effective medicines to cure various illnesses. Over-the-counter medication and unregulated use of antibiotics have helped evolve a set of multi-drug-resistant strains. This problem has catapulted into a grave concern relating to bacterial antibiotic resistance for overcoming infectious diseases [1]. Nosocomial infections are a channel of antibiotic drug resistance [2]. *P. aeruginosa* derived nosocomial infections have developed resistance to all commercial antibiotics [3]. Medicinal plant-based traditional medicine has emerged as active pharmaceutical ingredients in modern medicines. Now world over, the focus is shifting towards Pharmacognosy – drug discovery science from plant and plant-based sources.

It is not surprising to note that there have been several drugs molecules that have a natural origin. Some of the few well-known drugs from plant sources are Codeine and morphine [analgesic] from *Papavera somneferum*, Digitalin, Digitoxin and Digoxin [cardiotonic] from *Digitalis purpurea*, Menthol [rubefacient] from *Metha sp*., Nicotine [insecticide] from *Nicotiana tabacum*, Papain [mucolytic] from *Carica papaya*, Salicin from *Salix alba*, Vinblastine and Vincristine [antitumor] from *Catharanthus roseus* have been commercially successful to

date. Euphorbia species is notably the largest among blossoming plants in the world based on its approximately 8000 species. Christ's thorn or Crown of thorns are the other local names for *Euphorbia milli*. The various subspecies of the Euphorbiaceae family have well-documented traditional medicinal uses. Our Indian traditional medical systems like Ayurveda, Siddha and Unani have also utilised *Euphorbia hirta* and *Euphorbia polycarpa*. Since it is widely used in traditional, folklore medicine its dosage or other information needs to be scientifically validated.

Euphorbia milli is therapeutically indicated in the treatment of warts, abdominal swelling, cancer and hepatitis. Subsequent US patents also exist for Euphorobiaceae family based drug molecules like anti-cancer compound from *Euphorbia obesa* leading to US patent number 6923993 [2005], anorectal colonal disease treatment from *Euphorbia prostrata* leading to US patent number 2006/0198905 A1 in the year 2008, tumor inhibition from *Euphorbia antiquorum* leading to the US patent number 2003/0165579 A1 in the year 2002 to name a few [4]. The presence of secondary metabolites in plants is mostly responsible for the various beneficial activities. The same is attributed to the climatic condition, the age of the plants, the soil composition, water and nutrient availability, demographic location, and others, producing a wide variety of bioactive compounds. Haleshappa et al. have found tannins, terpenoids, flavonoids, steroids, betacyanin & anthocyanins in ethanol extract of *Euphorbia milli.* They have also concluded that amino acids, proteins and cardiac glycosides can only be found in the ethanolic extracts [5]. *Euphorbia balsamifera* leaves crude extract bioactive composition was categorised into cardiac glycosides, saponins, tannins, terpenoids, steroids balsam and flavonoids. The TLC of the crude extracts yielded 2, 3 and 4 spots respectively for chloroform, petroleum ether and ethanol extracts [6].

Using this study, we go through the various processes viz., extraction and screening leading to identifying the antimicrobial compounds of the leaves and flowers of *Euphorbia milli* against various pathogens. Researchers have already reported bio-active compounds from its bark. However, compound isolation and docking studies are found to be lacking. Hence the need for the present study.

Material and Methods

Collection of sample and preparation: The first step is to collect the fresh leaves and flowers of *Euphorbia milli* from VIT plant nursery, VIT, Tamilnadu. This was followed by washing the fresh leaves and flowers with sterile water and shade drying at room temperature. This was followed by grinding to a fine powder using a commercially available electrical blender.

Extraction of plant samples: Finely ground [50 g] powder of plant material was extracted in a soxhlet extractor for 8h with *n*-hexane and methanol, making sure that the solvent's boiling point is not exceeded or else continued till the sample becomes colorless. The extracts thus obtained were filtered through Whatman filter paper No.1 and subjected to reduced pressure concentration. Finally, the dried extracts were stored at 4°C for further experimental analysis [4].

Crude extract screening for phytochemicals: We proceeded with screening the extracts for the presence of various phytochemicals using standard protocols [5]. Phytochemicals screened were proteins, carbohydrates, quinones, terpenoids, steroids, cardiac glycosides, saponins, tannins, alkaloids, flavonoids and phenols.

Terpenoids: *2* ml of chloroform was mixed with 1ml of each [n-hexane and methanol] extract and mixed within a test tube. We then added 3 ml of concentrated sulphuric acid [H_2SO_4] to form a layer. If at the interface there is reddish-brown colouration it indicates the presence of terpenoids.

Quinones: we treat 1 ml of each plant extract

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with an equal amount of concentrated H_2SO_4 $_{[1ml]}$. The formation of red color indicates the presence of quinines.

Phenols: To 2 ml of distilled water 1 ml of extract was added. Then 10% ferric chloride few drops [$FeCl_3$] were added. The appearance of blue-green color indicates the presence of phenols.

Flavanoids: Few drops of dilute NaOH [sodium hydroxide] is mixed with 1 ml of extract. The intense yellow-colored plant extract becomes colorless in addition to few drops of dilute H2SO4, indicating flavonoids.

Tannins: To 0.5 g of solvent-free extract, add 1 ml of 5% ferric chloride and observe for bluish-black or greenish-black precipitation.

Saponins: 1 ml of extract was mixed with 2 ml of distilled water, shaken properly and allowed to stand for 10 min. Formation of 1 cm layer of foam lasting for more than 10 min, indicates the presence of saponins.

Glycosides: To 5ml of plant extract, 2 ml of glacial acetic acid containing a few drops of $Fecl_3$ solution was mixed. The appearance of a brown ring at the interface on the addition of 1 ml of conc. H_2SO_4 is the confirmation.

Steroids: About 10 ml of chloroform was dissolved with 1 ml of extract and then an equal volume of concentrated H_2SO_4 was added along the sides of the test tubes. The upper layer turned red and when H_2SO_4 was added, yellow color with green fluorescence was noticed.

Alkaloids: 1 ml of extract was mixed with 2 ml of reagent [2g Iodine + 6g KI +100 ml water] a positive test is indicated by the formation of red-brown precipitation.

Thin layer chromatography: This is the primary confirmation test to screen various components of the plant extracts on the chromatogram. The chromatogram was developed on the silica-coated TLC plate by spotting plant extracts [4,6]. The developing solvents used were toluene, ethyl acetate, methanol [4:0.5:0.5] for methanol extracts and for *n*-hexane extracts, hexane and ethyl acetate [4:1]. The separated bands were visualized under UV irradiation, as seen in Figure 3. The Rf values details are given in Table 4.

Antimicrobial assay: Using 0.1 ml of culture broth containing 1×10^6 cells/ml of pathogenic organisms in agar well diffusion method, we assessed the antimicrobial activity of the plant extract. On Mueller-Hinton agar plates, the pathogen was introduced using sterile cotton swabs to swab the surface. We punched five wells at least 30 mm apart in each agar plate using a sterile cork borer. Then we introduced various concentrations of the extracts [10µg/ml to 40mg/ml] into the wells in the plates. Ampicillin served as a control in the agar plates and was incubated at 37°C for 24-48h [7,8]. The resulting zones of inhibition were measured.

GC-MS analysis: The equipment used was a Clarus 680 Perkin –Elmer [Auto system XL] Gas Chromatograph coupled to a mass detector Turbo mass gold –Clarus 600 Perkin Elmer Turbo mass 5.1 spectrometers with an Elite –1 [100% Dimethyl poly siloxane]. The dimension of the capillary column was 30m x 0.25 mm ID x 250µm.

For 2 min, we maintained a temperature of 600°C, after which we raised the temperature by 100°C/min up to 3000°C, and maintained for 6 min. 2500°C was maintained in the injection port and 1ml/min was the Helium flow rate. We inject samples in split mode of 10:1 and 70eV ionization voltage with Mass spectral scanning from 50-600 [m/z]. Solvent Delay= 2.00 min, Transfer Temp= 230°C, Source Temp= 230°C.

GC-MS spectrum interpretation was done using NIST [National Institute of Standards and Technology] database Ver.2.1. Unknown compounds are compared with the database of known compounds. We identify the structure of the components of the test materials along with their name and molecular weight. Interpretation of GC-MS chromatogram was done using online bioinformatics software Molinspiration

and Molsoft L.C.C.

In Silico Analysis

Receptor 3d structure: Penicillin-binding proteins [PBP] were selected as one of the means in this investigation against microorganisms. We obtained the PBP structures from Research Collaboratory for Structural Bioinformatics [RCSB] and Protein databank [PDB].

We selected 3MZD with a crystallographic structure of high resolution [1.90Å] for *Escherichia coli*. Similarly, the structure for *Pseudomonas aeruginosa* [3OC2], *Salmonella typhimurium* [1NRF] and *Staphylococcus aureus* [1TVF] were attributed to the respective crystallographic structure of 1.85Å, 1.97Å, 2.50 Å, and 2.00 Å respectively of high resolution. Before proceeding further, we removed water molecules and co-crystallized ligands from the [3D] atomic coordinate file, while adding hydrogen atoms to the target protein molecule.

Homology modeling and structure validation: When the PBP has no significant crystal structure, we do not get a three- dimensional [3D] model of the target. Therefore we use Computational modeling for 3D structure prediction of proteins. Homology modelling was followed as per the standard guidelines [9]. The PBP sequence of *Streptococcus* sp, *Shigella* sp. and *Klebsiella* sp. in FASTA format was obtained from the UNIPROT database [10]. The 3D coordinates were constructed using I-TASSER using the FASTA sequences. This server I-TASSER is a virtual platform for predicting the function and structure of proteins. Iterative template fragment assembly simulations along with LOMETS are used to build 3D models after matching with BioLiP – Protein function database.

The least confidence score [c-score] which can range from -5 to 2 signifies the quality of the model by I-TASSER. Higher C-score signifies a high confidence model and vice-versa.

Further, the model quality evaluation was done using PROCHECK [11] server. This takes into consideration disulphide bonds, hydrogen bonds, non-bonded interactions, residue-by-residue analysis, planarity, chirality, dihedral angles, covalent geometry, stereochemical parameters, and parameter comparisons. While the backbone conformation of the angles [Phi and Psi] is accomplished using Ramachandran plot [12] as shown in Figure 1.

In similar research, the homology modelling of Endoxylanase from *Trichoderma pseudokoningii* was performed using PROCHECK, proSA & Verified_3D [20].

3d structure of plant active compounds: From the flowering plant *Euphorbia milii* six compounds were isolated and the structures obtained through GC-MS results were converted into 2D structures using ACD/ChemSketch version 12.01. All the ligand molecules were converted into 3D structures using Corina online server. Energy minimization for all the ligand coordinates was established using Chimera 1.8 [9,15]. The final 3D structures of the compounds are shown in Fig 2.

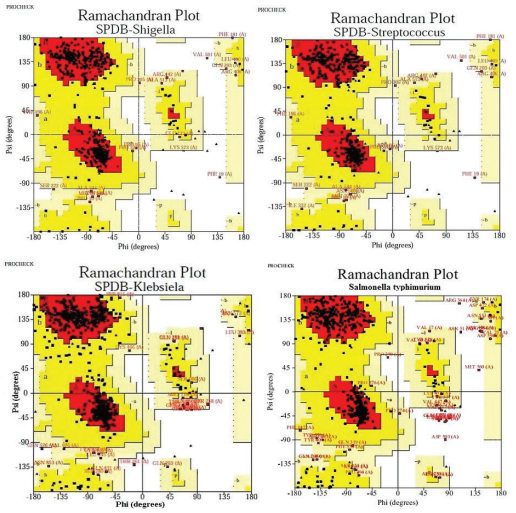

Fig 1: Ramachandran plot from PROCHECK showing the φ–ψ distribution for *Shigella* sp., *Streptococcus* sp, *Klebsiella* sp., and *Salmonella typhimurium.* All Regions viz., Most favored; additionally allowed; generously allowed, and disallowed are seen.

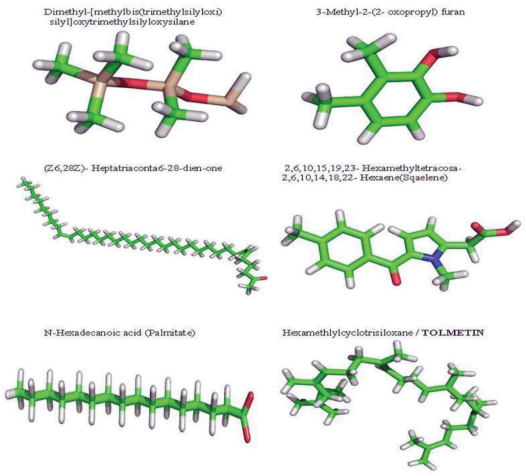

Figure 2: 3D structure of the Ligand co-ordinates

Predicting the active site and molecular docking: Prediction of the active site of the PBP chosen for our study was established with the help of Q-site finder, where protein-ligand binding sites are predicted using an energy-based method [11]. Protein preparation was followed as per the standard protocol laid down by Makesh Raj et al. [13]. The predicted ligand binding sites for the PBP are given in Table 2. iGEMDOCK was used for molecular docking simulation [14] version 2.1. Genetic Algorithm-Local Search [GA-LS] under standard conditions was used for molecular docking simulations. For visualizing the ligand-protein interactions, PyMOL program was used.

Drug scan: To prove the likeness to commercial drugs, we used Molinspiration server, which considers various parameters such as Bioactivity score and Molecular properties of the ligand co-ordinates [14]. This was done to ascertain coherence with Lipinski's Rule of 5 for any likely drug candidate [15]. Drug likeness results were shown in Table 3.

Results and Discussion

Once the plant extract is obtained for both the solvents, drying it completely for further analysis is the next step. The methanol extract of *Euphorbia milli* was vicious, dark reddish-brown in color and the hexane extract was sticky and

Table 1: Penicillin Binding Proteins Active Site Details

S.No.	PDB ID	Microorganism	Details of the Active Site
1	3MZD	*E. coli*	ARG304, ARG302, PRO301, PHE262, ARG261, PRO10, MET8, THR7, LYS6, and ILE5.
3	3OC2	*Pseudomonas* sp.	LEU180. GLY177, GLU176, ARG175, PHE167, GLY166, HIS163, ALA162.
4	1NRF	*Salmonella* sp.	MET 447, SER 448, SER449, SER450, SER525, ILE526, ARG527, LEU528, GLY538, LYS539, THR540.
5	1TVF	*Staphylococcus* sp.	TYR 291, THR 265, ASP 264, SER 262, ARG186, GLU183, ALA 182, GLY181, THR180, ASN141, SER116, LEU115, GLU114, LYS78, SER75, ALA74, ASN72.
6	Model 1	*Shigella* sp.	GLU456, GLY455, PRO454, VAL453, PRO443, ARG442, TYR441, ARG430, THR389, LYS387, ALA17, HIS16, GLY384, ASN18, ARG382, SER21, SER381, PHE383, TRP22.
7	Model 2	*Streptococcus* sp.	GLU158, ILE155, ALA154, ALA151, GLN127, THR126, ALA124, SER123, LEU119, SER118, PHE110, ARG105, ASP102, ILE101, GLY100, ARG99, HIS98, ASP97, PHE95, ARG94, ASP92.
8	Model 3	*Klebsiella* sp.	GLU389, ILE386, VAL385, GLN384, GLN383, GLN382, GLN381, LEU380, LEU379, ARG378, LEU377, VAL376, ASP345. LEY344, THR232, ALA231, THR228, LEU 224, ASP227.

green in color. *Euphorbia milli's* methanol extract had almost all the phytochemical constituents except proteins whereas hexane extract showed the presence of quinines, steroids, and terpenoids. The phytochemical profile has been presented in Table 4. The methanol extract of the leaves of *Emblica officinalis* was tested for their antimicrobial effect against gram-positive, gram-negative bacteria and yeast. The compounds were also identified using GC-MS [19].

The need for thin-layer chromatography is to visualize the separation of phytochemical constituents present in plant extracts. To separate the components present in plant extracts various solvent systems were employed. However, toluene: ethyl-acetate: methanol [4:.5:0.5] gave good separation for obtained methanolic extracts, whereas hexane: ethyl acetate [9:1] resulted in good separation of hexane extracts. The Rf value was calculated and has been presented in Table 1 and the separation of phytochemical agents has been presented in Figure 1.

Table 2. Drug likeness and Bioactivity values of *Euphorbia milli* crude methanol extract.

Compound name	HBA	HBD	Log P	Drug likeness	Molecular weight	Molecular formula	Molar Refractivity [in cm^3]
Dimethyl-[methyl-bis[trimethylsilyloxi] silyl]oxy-trimethyl silyloxysilane	4	0	6.51	-1.31	384.15	$C_{12}H_{36}O_4Si_5$	108.21 ± 0.3
Z,Z-6,28- Heptatria contadien-2-one	1	0	14.9	-0.66	530.54	$C_{37}H_{70}O$	173.61 ± 0.3
N-Hexadecanoic acid[Palmitate]	2	1	6.60	0.95	256.24	$C_{16}H_{32}O_2$	77.73 ± 0.3
3-Methyl-2-[2- oxopropyl] furan	2	0	1.76	-1.67	138.07	$C_8H_{10}O_2$	37.58 ± 0.3
[6E,10E,14E,18E]-2,6,10,15, 19,23- Hexamethyl tetracosa-2,6,10,14,18,22-Hexane [Sqaelene]	0	0	12.5	-0.99	410.39	$C_{30}H_{50}$	140.43 ± 0.3
Hexamethlylcyclotrisiloxane	3	1	2.15	0.95	257.11	$C_{15}H_{15}NO_3$	59.26 ± 0.4

*HBA- Hydrogen Bond Acceptor, ** HBD- Hydrogen Bond Donor

Table 3. Phytochemical profiling of the plant extract of *Euphorbia milli*.

S No.	Phytochemical Tests	Methanol extract	Hexane extract
1	Alkaloids	+	-
2	Tannins	+	-
3	Terpenoids	+	+
4	Phenol test	+	-
5	Steroids	+	+
6	Carbohydrates-Fehling A	-	-
7	Carbohydrates-Fehling B	+	-
8	Quinones	+	+
9	Flavanoids	+	-
10	Proteins	+	-
11	Saponin	+	-

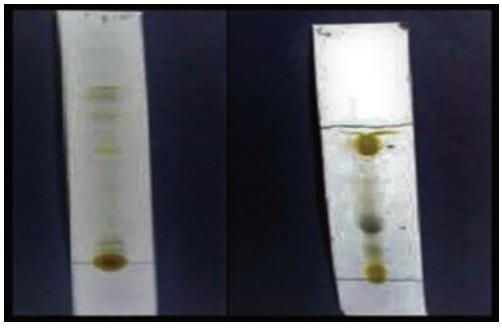

Figure 3: Methanol extracts of *Euphorbia milli* undergoing thin layer chromatography

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Table 4: *Euphorbia milli* methanol extracts thin layer chromatography data

Euphorbia milli Methanol Extract	Distance Covered by the spots [in cm]	Rf values	Distance traveled by solvent
	0.25	0.0625	4.0 cm
	0.50	0.125	
	2.5	0.625	
	3.3	0.825	
	3.65	0.912	
	3.90	0.975	

We varied the concentration from 10-40mg/ml and determined the minimum inhibitory concentration. Results point to the antimicrobial effect in methanol extract against the tested pathogens. At the same time, hexane extracts were not found to be effective antimicrobial agent.

It was found that the antimicrobial effect was comparable to the standard antibiotic ampicillin based on a similar zone of inhibition. Methanol extract of *Euphorbia milli* was very effective against *Staphylo-coccus aureus* followed by *Pseudomonas aeruginosa* and *Streptococcus pneumoniae* as shown in Table 5.

The GC-MS data of the methanol extract of *Euphorbia milli,* along with its bioactivity score is shown in Table 6. From the six compounds identified in crude Methanol extract of *Euphorbia milli* two compounds can be regarded as potent drug agents, which include 3-Methyl-2-[2-oxopropyl]furan and Hexamethyl-cyclotrisiloxane.

Table 5: Minimum inhibitory concentration [mm] of methanol extracts of *Euphorbia milli* against clinical pathogens.

Concentration [mg/ml]	*E. coli*	*Staphylococcus aureus*	*Streptococcus pneumonia*	*Pseudomonas aeruginosa*	*Shigella dysenteriae*	*Salmonella typhimurium*	*Klebsiella pneumoniae*
10	14	13	9	9	10	9	11
20	14	13	10	10	10	10	11
30	14	14	11	11	10	11	11.5
40	14	15	12.5	14	12	12	12
Ampicillin	17	20	18	19	17	17	17

Docking analysis

iGEMDOCK was used for the docking of the six ligand molecules onto the active site of the PBP's [13,17]. The docking score is then used to arrive at the best pose of the ligand molecules. The docking poses for the above-mentioned interactions of the ligand with the PBP of all the microbes are given in figures 4-10. Similarly, docking tools are being successfully implemented to prove the antidepressant effect of natural compounds in blocking the effect of serotonin transporter (SERT) responsible for depression [18].

Based on the six compounds screened, Hexamethlylcyclotrisiloxane/Tolmetin has the best binding affinity due to its docking score; the details of which are given in Table VII. It is

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Table 6. Bioactivity and drug-likeness values of crude methanol extract of *Euphorbia milli.*

Compound name	HBD	HBA	Log P	Drug like-ness	Molar Refractivity [in cm^3]	Molecular weight	Molecular formula
Dimethyl-[methyl-bis[trimethylsilyloxi]silyl]oxy-Trimethyl silyloxysilane	0	4	6.51	-1.31	108.21 ± 0.3	384.15	$C_{12}H_{36}O_4Si_5$
Z,Z-6,28-Heptatria contadi-en-2-one	0	1	14.9	-0.66	173.61 ± 0.3	530.54	$C_{37}H_{70}O$
N-Hexadecanoic acid[Palmitate]	1	2	6.60	0.95	77.73 ± 0.3	256.24	$C_{16}H_{32}O_2$
3-Methyl-2-[2-oxopropyl]furan	0	2	1.76	-1.67	37.58 ± 0.3	138.07	$C_8H_{10}O_2$
[6E,10E,14E,18E]-2,6,10,15, 19, 23- Hexamethyltetraco-sa- 2,6,10,14,18,22-Hexaene [Sqaelene]	0	0	12.5	-0.99	140.43 ± 0.3	410.39	$C_{30}H_{50}$
Hexamethlylcyclotrisiloxane	1	3	2.15	0.95	59.26 ± 0.4	257.11	$C_{15}H_{15}NO_3$

*HBA – Hydrogen Bond Acceptor, ** HBD – Hydrogen Bond Donor

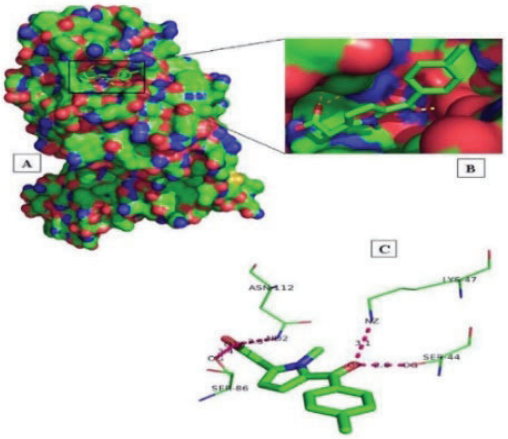

Figure 4: *E.coli* [3MZD] Docking results. [A]. Tolmetin in the active site of *Ecoli*'s PBP. [B]. Binding site close-up view. [C] Tolmetin's interactions and bond distance in Å units is seen.

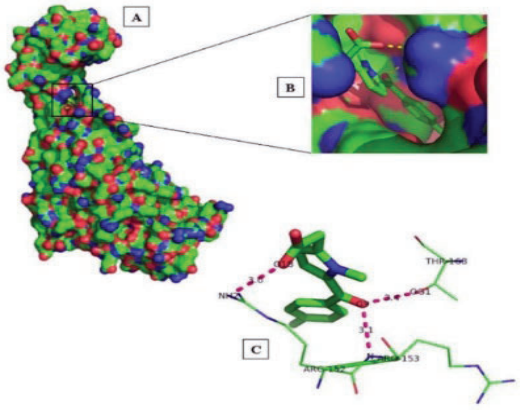

Figure 5: *Pseudomonas* docking results [A]. Tolmetin binding with *Pseudomonas* PBP. [B]. PBP [3OC2] binding site focus. [C] Tolmetin's interactions and bonding distance in Å units is seen.

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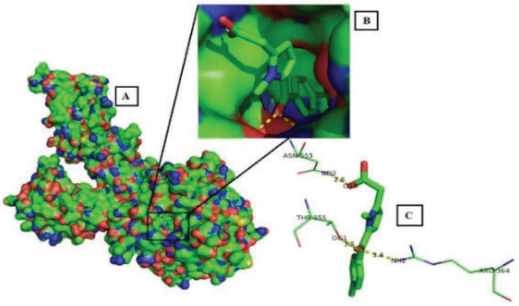

Figure 6: *Salmonella* docking results [A]. Tolmetin binding with *Salmonella* [1NRF] PBP. [B]. The focus of Binding [C] Bond length of the interaction in Å units for all interactions is visible.

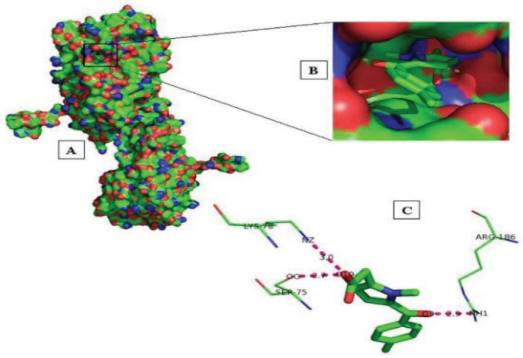

Figure 7: *Staphylococcus* docking results [A]. Tolmetin binding with *Staphylococcus* PBP [1TVF]. [B] Binding focus [C] Interaction of Tolmetin with PBP and binding distance in Å is seen.

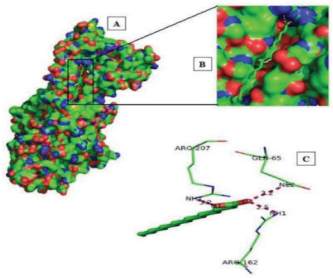

Figure 8: *Shigella* docking results. [A] N-Hexadecanoic acid binding with *Shigella* PBP. [B]. Binding site focus. [C] Various interactions between N-Hexadecanoic acid and PBP with bond distance in Å units is seen.

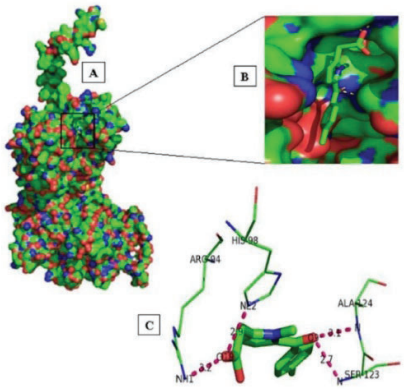

Figure 9: *Streptococcus* Docking results [A] Tolmetin binding with *Streptococcus* PBP. [B] Binding site focus [C] Interactions between Tolmetin and PBP with bond distance in Å units is also shown.

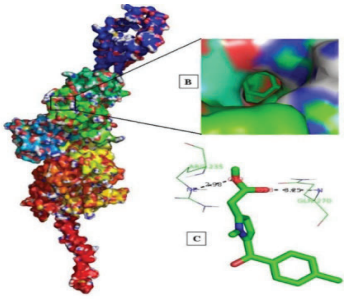

Figure 10: *Klebsiella* docking results [A]. Tolmetin binding with *Klebsiella* PBP. [B]. Binding site focus [C] Tolmetin interaction with PBP. View of the surface model of Protein with the interactions of amino acids residues and hydrogen-bond networks and bond distance in Å units is also shown.

found to have interaction with the entire targeted PBP's except that of *Shigella dysenteriae.* Binding energy and strong hydrogen bonding interactions for the docked complexes with the best docking score were tabulated in Table VII.

(Interchange the tables)

From Table 8 it is confirmed that Tolmetin is found to be very effective against *Staphylococcus*

aureus [-90.20 Docking score] forming three hydrogen bond interactions with key residues SER 75, LYS 78, and ARG 186, all three of them predicted active sites. Tolmetin is equally effective against *Pseudomonas* [-86.78 Docking score] and *Streptococcus* [-85.63 Docking score], forming three [ARG 152 – predicted active site residue, ARG 153, THR 168] and Four [ARG 94, HIS 98, SER 123, ALA 124 all predicted active sites residues] hydrogen bonds

Target PBP / Ligand	*E. coli*	*Klebsiella sp.*	*Pseudomonas sp.*	*Salmonella sp.*	*Shigella* sp.	*Staphylococcus* sp.	*Streptococcus sp.*
Dimethyl-[methyl- bis[trimethylsilyloxi]silyl]oxy- trimethylsilyloxysilane	-49.73	-51.64	-50.76	-46.10	-50.34	-48.28	-50.66
[Z6,28Z]- Heptatriacontа6-28-dien-2-one	-97.02*	-90.02*	-88.50*	-81.26	-85.74*	-79.19	-88.70*
N-Hexadecanoic acid [Palmitate]	-68.16	-78.19	-70.78	-75.85	**-79.16**	-73.51	-81. 20
3-Methyl-2-[2- oxopropyl] furan	-64.83	-66.56	-67.92	-60.73	-62.63	-74.47	-69.14
2,6,10,15,19,23- Hexamethyl tetracosa-2,6,10,14,18,22- Hexaene [Sqaelene]	-68.82	-69.35	-81.49	-64.74	-71.86	-71.98	-81.60
Hexamethlylcyclotrisiloxane / Tolmetin	**-78.02**	**-86.38**	**-86.78**	**-81.96**	-77.13	**-90.20**	**-85.63**

Values marked '' do not have any interactions with the target protein despite having a higher docking score. The second-best score is therefore considered.* ***** Docking score is in Kcal/mole

Table 8. Docking Results

S.No	Protein	Ligand	Total Energy	VDW	H Bond	Electrostatic
1.	*E. coli* [3MZD]	Tolmetin	-78.02	-65.40	-10.65	-1.96
2.	*Pseudomonas* [3OC2]	Tolmetin	-86.78	-75.25	-9.29	-2.24
3.	*Salmonella* [1NRF]	Tolmetin	-81.96	-63.05	-17.01	-1.90
4.	*Staphylococcus* [1TVF]	Tolmetin	-90.20	-69.16	-19.72	-1.32
5.	*Shigella*	N-Hexadecanoic acid [Palmitate]	-79.16	-53.44	-19.10	-6.62
6.	*Streptococcus*	Tolmetin	-85.63	-67.10	-14.45	-3.25
7.	*Klebsiella*	Tolmetin	-86.38	-82.83	-3.44	-0.11

Note: *Values rounded off to the nearest two digits after the decimal point.* ***** Docking score is in Kcal/mole

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respectively.

Nitrogen in positions Z, H1, H2, and E2 and Oxygen atoms in positions G and G1 are the interacting atoms concerning the PBP of the above-mentioned three pathogens. While from the ligand side [Tolmetin] only Oxygen atoms are being involved in the interactions at positions 9, 18, and 19, respectively. The interacting atoms and their H bond length are given in Table 9.

Table 9. Post-docking interaction analysis

S.No	Protein	Interactions		Bond Length[A°]
	E.coli [3MZD] - Tolmetin	SER44	**O**G – **O**9	2.6
		LYS47	**N**Z – **O**9	3.1
		SER86	**O**G – **O**18	3.4
		ASN112	**N**D2 –**O**18	3.3
	Klebsiella -Tolmetin	GLN 270	**N** – **O**18	3.25
		ARG235	**N**L – **O**19	2.98
	Pseudomonas [3OC2] - Tolmetin	ARG 152	**N**H2– **O**18	3.0
		ARG153	**N** – **O**9	3.1
		THR168	**O**G1 – **O**9	3.4
	Salmonella [1NRF] - Tolmetin	LYS 405	**N**Z – **O**18	2.6
		SER 450	**O**G – **O**9	3.5
		THR 452	**O**G – **O**18	2.5
		TRP 488	**N**E1 – **O**19	3.3
	Shigella dysenteriae – N-Hexadecanoic Acid	GLN 65	**N**E2 – **O**3	3.2
		ARG 162	**N**H1 – **O**3	2.5
		ARG 207	**N**H1 – **O**1	3.2
	Staphylococcus [1TVF] - Tolmetin	SER 75	**O**G – **O**19	2.7
		LYS 78	**N**Z – **O**19	3.0
		ARG186	**N**H1 – **O**9	2.9
	Streptococcus pneumonia - Tolmetin	ARG 94	**N**H1 – **O**19	3.2
		HIS 98	**N**E2 – **O**19	2.9
		SER123	**N** – **O**9	2.7
		ALA124	**N** – **O**9	3.1

Conclusion

The onset of the era of multiple drug resistance has pushed us to search for new active drug molecules having diverse chemical structures and mechanisms of action. Pharmacognosy has helped by providing various natural compounds not limiting to antibacterial and antifungal agents to be used as likely drug candidates. Secondary metabolites in plants account for various categories such as saponins, flavonoids, alkaloids, terpenoids, and tannins, which exhibit antimicrobial activity. The present work focuses on methanol extract of *Euphorbia milli*. Both antibacterial assay and interpretation of GC-MS results have proved the presence of antibacterial activity. The same was further validated using molecular docking studies. We were able to identify the target site, which is the Penicillin Binding Proteins for the various pathogens. The ligand molecules which were obtained from GC-MS were used during the interaction with the amino acids in the microbial Penicillin Binding Proteins. The Ligand – Protein interaction occurred in the active binding site, which was possible through strong bonding interactions. Post docking analysis helps us understand the mechanism by which these likely drug candidates can function. From the experimental and *Insilico* analysis of the isolated bioactive compounds of *Euphorbia milli* we can zero in on Tolmetin to be a potential inhibitor against the PBP of the bacterial pathogens under study.

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