Design, *in-silico* study and Synthesis of dipeptides for biological evaluation as an Antimicrobial Agent

Anjali Saini[1], Aiman Sifat[2*], Shweta Sharma[3], Shruti Gupta[4]
[1]Metro College of Health Science & Research, Delhi-NCR, Greater Noida, 201310, India
[2] HRIT University, Delhi –NCR, Ghaziabad, 201003, India*
[3]Dr. Akhilesh Das Gupta Institute of Professional Studies, GGSIP University, Delhi,110053, India
[4]DJ College of Pharmacy, Delhi-NCR, Modinagar, 201204, India

[1]anjalihrip1997@gmail.com, [2]sifat21aiman@gmail.com, [3]Shweta.lav@gmail.com, [4]sg4844107@gmail.com

***Author for correspondence**
Dr. Aiman Sifat
Address: HRIT University, 08 km milestone, Ghaziabad (U.P), 201003, India
Phone: +91-8979729339

E-mail addresses: sifat21aiman@gmail.com, anjalihrip1997@gmail.com

Abstract
Antimicrobial resistance has now become a global burden from several years. To prevent drug resistance, there is a need to develop safer and more potent peptide lead. Oxidoreductase has been chosen as a target for our study, crucial for decreasing antimicrobial resistance apoptotic activity. The purpose of this research work is to determine the peptides of varying length were selected. Swiss Dock online tool was used for docking (PDB code: 3H4K) along with Chimera and Discovery studio visualizer for detailed investigation of 3D interactions. In-vitro antioxidant capacity by the Phosphomolybdenum method has been used to evaluate complexion capacity of the IC_{50}value. FT-IR, DC-MS, and ^1H NMR of lead peptides were recorded and analyzed. Also acute oral toxicity test were conducted by using the dose limit 2000 mg/kg body weight and to explore the minimum inhibition concentration of lead peptides evaluated through in-vitro antimicrobial effect using *Streptococcus aureus* (ATCC 29213) and *Pseudomonas aeroginosa* (ATCC 27853) strains. Proline-Methionine have shown the most promising peptide lead with ΔG value of - 8.24 Kcal/mol (full energy: -2865.41) against oxidoreductase target. The Proline-Methionine has shown better in-vitro potency with IC_{50} value 8μg/ml in Phosphomolybdenum assay. The dipeptide has shown potent antimicrobial activity using *Pseudomonas aeroginosa* (ATCC 27853) strain with MIC value of 4μg/ml using broth dillution assay. The same compound was very well characterized with FT-IR spectroscopy, ^1H NMR, DC-MS spectrometry data. Also according to acute toxicity study, the experimental design on rodents by the oral administration of Proline-Methionine (dipeptide) for 14 days in female rats was identified as slightly toxic under category-4.

Key words- ***Antimicrobial, Antioxidant, Docking, Frequencies, Peptides.***

1. Introduction

Peptide bonds, also known as amide bonds or peptide bonds, bind the individual amino acids of the subunit together. Peptides are organic substances made up of two or more amino acids connected by amide bonds between the carboxyl groups of neighboring amino acids and the amino groups of the adjacent amino acids ***(Miller et al 2016).*** A residue is a name for one amino acid unit in a peptide. The crucial part that peptides play in determining the specificity, magnitude, and quality of both humoral antibody and cellular (cytotoxic and helper T cell) immune responses has been made clear by analytical biochemistry and synthetic peptide-based chemistry ***(Boone et al 2018)***. Our own body makes peptides, but experimental based design peptides can be mimicking the body's peptides.

Antimicrobial resistance means when antibiotic not work longer to treat or prevent from microbial infection. An antimicrobial resistance problem has been ovehelmed during the penicillin discovered. According to the world health organization estimated that around 1.27 million deaths occur associated with the estimation in around 5.20 million deaths compare with the AIDS patients (Jonathan 2022). This is mainly due to the lack of patient's compliance and mutation transfer ***(Mc et al 2018).***

Oxidoreductase enzyme involves in the detoxification in liver, oxidative phosphorylation, glycolysis etc. oxidoreductase enzyme having a better biodegradability, efficiency as well as specificity. This enzyme helps in treatment, diagnosis the microbial infection in living organisms. Oxidoreductase decreases bactericidal infection in host cell and apoptotic activity, also increases the macrophages activity and boost the immune responses ***(Kareem et al 2021).***

Acute toxicity is the term used to describe adverse reactions that occur within 24 or 4 hours of inhalation exposure after single or multiple doses are administered orally or topically. Between acute and chronic toxicity is a distinction. Chronic toxicity refers to negative health effects that develop over time after repeated exposure to a substance, frequently at low doses and for a long time (months or years). Human subjects for acute (or chronic) toxicity studies are typically regarded as unethical. The acute toxicity method is based on biometric analyses using evenly spaced fixed doses to allow substances to be classified for classification and hazard assessment purposes. Utilizing his LD50 data from domestic and international literature, this method, which was first introduced in 1996, has undergone extensive in vivo validation ***(Annonymus et al 2002).*** Because carboxymethyl cellulose sodium, an anionic derivative of carboxymethyl cellulose, is sodium salt, it was used in our study instead of sodium SCMC suspension. It is frequently used in pharmaceutical formulations that are oral, ophthalmic, injectable, and topical. It is primarily utilized as a binder or matrix former for solid dosage forms ***(Vasileious et al 2020).***

The global burden of antimicrobial resistance was found in 2019 to compare with that of HIV/AIDS. Molecular docking of potential inhibitors of the influenza H7N9 virus has been reported to pose a global threat, and molecular docking has also been shown to be effective against drug resistance ***(Liu et al 2015).*** Dioxin-scavenging peptides were reported through docking-based screening those select shorter-chain peptides using an inexpensive docking method ***(German et al 2013).*** *In -silico* screen for HNF-1a receptor ligands was evaluated and predicted. HNF-1a is a transcription factor that regulates metabolism to glucose and measures hydrogen bond distances, comparing it to resveratrol and aspirin ***(Jiaorong et al 2019).*** Docking studies were used to evaluate selected antimicrobial peptide against pathogenic bacterial species. Peptides are made up of different amino acid sequences. Interpretation is done by FT-IR, UV-VIS, NMR, and laser-Raman spectroscopy studies on the antidiabetic drug meglitinide ***(Tuba et al 2018).*** Monitor the metabolite secretion of tryptophan by metabolizing mutant E. coli using FT-IR and DC-MS ***(Naheed et al 2003)***. Determination of minimum inhibitory concentration against the strain *Candida albicans* was predicted ***(Acacio et al 1993).***

This research work was carried out to interpret the theoretical and experimental investigation of the dipeptides as the potent lead for antimicrobial agent along with its physiochemical characterization of synthesized compounds using spectroscopic analysis such as FT-IR, DC-MS, NMR and UV- Visible spectrometry. In addition the same dipeptides are subjected to acute toxicity studies and *in-vitro* antioxidant studies.

II Experimental Methods

2.1 Molecular structure preparing and activity prediction by *in-silico* method

The Configuration of the system in which the docking study was performed along with other online tools of drug design was running on a 2.00 GHz Dell Inspiron Laptop Intel ® Core ™ processor, 4.00 GB RAM, and 64-Bit window operating system. Molecular docking was performed with SWISS DOCK online tool (available at http://www.swissdock.ch/docking) the selective peptides based on preliminary results obtaining by the protein oxidoreductase of PDB code- 3H4K as a protein ligand, whereas the peptides were in the ligand selection ***(Maiia et al 2002).*** The PDB file was downloaded from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (available at https://www.rcsb.org/structure/3H4K). The peptide molecules were visualized and analyzed by the UCSF chimera tool (version 1.16, available at https://www.cgl.ucsf.edu/chimera/) and Biovia Discovery studio (available at https://discover.3ds.com/discovery-studio-visualizer-download), which is the software used to viewing and analyzing the modeling molecules.

Glycine-Aspartic acid, Glycine-Valine, Proline-Methionine, Alanine-Lysine, Aspartic acid-Tyrosine, Glycine-Alanine, Glycine-Glutamine, Proline-Tyrosine, Alanine-Phenylalanine and Aspartic acid-Cysteine, were selected from free source of various ligands molecules Pubchem (available at https://pubchem.ncbi.nlm.nih.gov/) in this study and subjected to SWISS ADME online tool to check the drug-likeness property of Lipinski rule of five along the pharmacokinetic property ***(Kandasamy et al 2022)***. Bioavailability of all dipeptides molecules were tested against permeability glycoprotein substrate along with the metabolic profile of drug against various cyto-chrome inhibitions (CYP1A2, CYP2C19, CYP2C9, CYP2D6, and CYP3A4) (available at http://www.swissadme.ch/). Molinspiration chemoinfromatics online software was used for all the dipeptides for predictions of various physiochemical parameters (available at https://www.molinspiration.com/cgi-bin/properties) along with the enzyme inhibition bioactivity scores as a general way of identifying the potent dipeptide lead ***(Hadda et al 2021).*** The same sets of all dipeptides were subjected to PASS WAY 2 DRUG Online tool to identify the activity against the possible adverse effects and toxic effects (available at http://www.way2drug.com/passonline/) ***(DA et al 2014).***

2.2 Synthesis of peptide molecules

All amino acid kit was purchased from CDH lab in powdered form and dried in nature. Varying length of peptides chain were synthesized though Liquid phase peptide synthesis method (LPPS) with pthalic anhydride as protecting agent, chlorophosphate ester reagent as condensing agent and vaccum distillation apparatus with pyridine as for deprotection of synthesized dipeptides and purified by column chromatography using silica gel as stationary phase and Methanol for crude, $_n$Hexane of ratio 100%, $_n$Hexane- ethyl acetate of ratio 75:25, $_n$Hexane- ethyl acetate of ratio 50:50, $_n$Hexane- ethyl acetate of ratio 25:75, ethylacetate of 100%, ethylacetate-methanol of ratio 75:25, ethylacetate-methanol of ratio 50:50, ethylacetate-methanol of ratio 25:75, methanol of 100%, methanol-water of ratio 80:20, methanol-water of ratio 60:40, methanol-water of ratio 40:60 and $_n$butanol-acetic acid-water of ratio 40:10:50 fractions of solvents work as mobile phase ***(Sharma et al 2022).*** TLC is used to determine the R_f value of all peptides with iodine chamber for visualization of spots. The melting point of selected peptides is measured through the Veggo instrument.

2.3 Physiochemical characterization of peptides by FT-IR, NMR, DS-MS

2.3.1 FT-IR Analysis

The FT-IR spectrum was done by SHIMADZU FTIR-AFFINITY 1 of dipeptide molecules (Proline-Methionine, Aspartic acid-Cysteine) and the spectra were recorded from IR Solution software in between 4000 to 500 cm^{-1} by KBR disc method of model number

2.3.2 NMR Analysis

The ^1H NMR known as a non-destructive analytical method which detect the chemical shift spectra in ppm of the dipeptide molecules (Proline-Methionine, Aspartic acid- Cysteine) was dissolved in DMSO-D_6 solvent by using BRUKER NMR of model number

2.3.3 DS-MS Analysis

The DC-MS spectra analysis was performed and recorded in Indian Pharmacopoeia Commission (IPC-Ghaziabad), Delhi- NCR; 2mg of dipeptides (Proline-Methionine, Aspartic acid- Cysteine) were dissolved in 10ml of methanol and filtered over micro porous membrane for DC-MS analysis with quadropole-TOF of model Axion DSA perkin elmer.

2.4 Biological Evaluations

2.4.1 In-Vitro Antioxidant activity (Phosphomolybdenum Method)

Phosphomolybdenum method is an *in-vitro* antioxidant activity measure the IC_{50} value at different specific concentration by using 0.1 ml of dipeptides (Proline-Methionine, Aspartic acid- Cysteine) with 1ml of reagent combined with sulphuric acid, sodium phosphate and ammonium molybdate. Tubes were boiling on water bath at 100 degree Celsius for one and half hour. After cooling the sample at room temperature the absorbance of dipeptides was measured at 695nm using software UV Probe of UV-Visible double beam spectrophotometer (Shimadzu-1800) and the IC_{50} were measured from respective %inhibition using graphical method. (P et al 1999) ***(Saini et al 2022).***

2.4.2 In-Vitro Antimicrobial assay of MIC prediction

As per the European Committee for Antimicrobial Susceptibility Testing (EUCAST)/CLSI, the broth dilution method was used to determine the minimum inhibitory concentration (MIC) of compounds against rapidly growing bacterial pathogens. Caution supplemented Mueller-Hinton broth II was used as a medium. In brief, 100 µL was added in well to serve as a sterility control well. Further, 50 µL were added to another well and served as a control well. Then, 50 µL of each dilution (2:1) of antibiotic, were added to the respective well and further inoculated with colonies of bacteria. The inoculation was performed in such a way so that it could contain 5×10^5 CFU/ml. The plate was incubated at 35-37^{0}C for 18-24 hrs. The concentration at which there is no visible growth of bacteria was observed was taken as MIC ***(Anonymous et al 2003).***

2.4.3 Acute Oral Toxicity Study on Rodents

2.4.3.1 Experimental animals

All the protocol on animal model was approved by IAEC of KIET School of Pharmacy with CPSCEA proposal no. IEAC/KSOP/2022-23/11 the female Albino rats were provides from the national institute of biologics (NIB), rats were placed under the condition of 23°C± 2 °C and 12 hours of light-dark cycle. And allow free access food and water. Female rats of weight 120- 300 g were taken for acute toxicity testing. They were house in plastic laboratory cages in ventilated room of animal house. Rats were acclimatized to this environment for only 7 days before receiving the experimental treatment.

2.4.3.2 Acute toxicity evaluation

Experiments to determined or assessment of LD_{50} values (lethal dose 50%) of dipeptides (Proline-Methionine, Aspartic acid- Cysteine) were designed according to the OECD guideline 423 (Annexure 2A) method (Anonymous et al 2002). Four hours before dosing, food was withheld, but the water was withheld. Dipeptides were uniformly suspended in 0.5% Sodium carboxy methyl cellulose by vortexing and sonicating the suspension for 5 minutes. Dipeptides were prepared using 0.5 percent sodium Carboxymethyl cellulose (low viscosity grade) to make the desired concentration for oral administration, sodium CMC used as a vehicle control. Four different concentrations of dipeptide drug (5, 50, 300, and 2000 mg/kg) has been administered to each group of 3 rats in a graded procedure through oral gavage. Three rats treated with the same dose of 0.5% SCMC served as controls. Changes in weight, behavior, and symptoms were carefully recorded daily after treatment.

III Results and Discussion

3.1 Interpretation of *in-silico* data

The Swiss ADME tool was used for all selected ten dipeptides Glycine-Aspartic acid, Glycine-Valine, Proline-Methionine, and Alanine-Lysine, Aspartic acid-Tyrosine, Glycine-Alanine, Glycine-Glutamine, Proline-Tyrosine, Alanine-Phenylalanine and Aspartic acid-Cysteine with physicochemical properties (molecular mass, number of heavy atoms, count aromatic heavy atoms, number of rotatable bonds, number of hydrogen bond donors, number of hydrogen bond acceptors, molar refractive index, total polar surface), lipophilicity (consensus log Po/w), water solubility (log S), all ADME properties (GI absorption, permeation through the blood-brain barrier, PGP substrate, inhibitors of cytochrome CYP1A2, CYP2C19, CYP2C9, CYP2D6, CYP3A4, skin permeation (log KP), Lipinski's rule drug similarity characteristics, bioavailability score, lead similarity and synthetic availability as multiple parameters and the results of all ten dipeptides were expressed in Table 1a and 1b. Further, the same set of compounds was subjected to Molinspiration chemo-informatics tools and the results are expressed in Table 2.

This tool has indicated that most of the dipeptides were filtered off with relative bioactivity scores as general enzyme inhibitors and found that only specific dipeptides. In addition, for all dipeptide molecules with isomeric SMILES notation, the PASS online Pass way-2 drug software tool was used to predict additional bioactivity of shorter to longer-chain dipeptides against specific targets antimicrobial resistance, the anticipated potential for unwanted toxic effects.

Table 3 shows the PASS activity value results. By comparing the Molinspiration data and PASS way 2 drug data , of all dipeptides were selected for further docking investigation, namely against the specific target oxidoreductase (3H4K). The results of docking study with ΔG value Kcal/mol are expressed in Table-4.

The detailed docking investigation of best two dipeptides molecule Proline-Methionine and Aspartic acid-Cysteine are tabulated in Table-4 and their best molecule docking interactions are clearly visualized in Figure 1-2.

The docking study was identified as Proline-Methionine with ΔG value of - 8.24 Kcal/mol (full energy: -2865.41), and Aspartic acid-Cysteine with ΔG value of - 8.30 Kcal/mol (full energy: -2890.90).

Among the ten dipeptides, the Proline-Methionine with ΔG value of - 8.24 Kcal/mol was found to be the most promising compound as potent antimicrobial agents as oxidoreductase inhibitor through *in- silico* method.

Table 1a: SWISS ADME Physiochemical characteristic features of peptides molecule. As from Table-1A, it has been observed that Proline-Methionine was identified as the best lead with the physiochemical properties, especially H-bond acceptor values with 3 less than 5 along with the TPSA as 78.42 which should be between 40-130 and having molecular weight 246.33 less than 500 Dalton as per Lipinski rule of five (Lipinski et al 2004).

Peptides	Physicochemical Properties								Lipophilicity Consensus	
	Mol. Weight	No. of Heavy Atoms	No. of Aromatic Heavy Atoms	No. of Rotatable Bonds	No. of H-Bond Donor	No. of H-Bond Acceptor	Molar Refractivity	TPSA A^2	Log P O/W	H2O Solubility Log S (ESOL)
Proline-Methionine	246.33	16	0	7	4	3	67.15	78.42	-0.38	1.04
Aspartic acid-Cysteine	236.25	15	0	7	6	4	52.94	-4.10	-1.72	1.80
Glycine-Aspartic acid	190.6	13	0	6	4	6	40.21	129.72	-2.25	2.62
Glycine-Valine	174.2	12	0	5	3	4	43.24	92.42	-0.86	1.10
Alanine-Lysine	217.27	15	0	8	5	4	55.57	118.44	-0.96	1.54
Aspartic acid-Tyrosine	296.28	21	6	8	7	5	71.53	149.95	-1.18	1.38
Glycine-Alanine	146.15	10	0	4	3	4	33.63	92.42	0.31	1.82
Glycine-Glutamine	204.18	14	4	6	4	6	45.02	129.72	-0.01	2.51
Proline-Tyrosine	278.31	20	0	6	4	5	76.46	98.65	1.56	0.54
Alanine-Phenylalanine	236.27	17	0	5	3	4	62.92	-1.79	1.37	0.39

Table 1b: SWISS ADME Pharmacokinetic profile and drug likeness characteristic of peptides molecule. As from table-1b, it has been observed that Proline-Methionine has shown CYP2D6 inhibitor having no hepatotoxic effect with high synthetic accessibility along with the absence of lead likeness.

Peptides	Pharmacokinetic profile									Drug Likeness			
	GI Absorption	BBB Permeant	PGP Substrate	CYP 1A2 Inhibitor	CYP 2C19 Inhibitor	CYP 2C9 Inhibitor	CYP 2D6 Inhibitor	CYP 3A4 Inhibitor	Log Kp (Skin Permeation) CM/S	Lipinski	Bioavailability Score	Lead Likeness	Synthetic Accessibility
Proline-Methionine	High	No	Yes	No	No	No	No	No	-10	Yes	0.55	No	2.99
Aspartic acid-Cysteine	High	No	No	No	No	No	No	No	-10.72	Yes	0.11	No	2.8
Glycine-Aspartic acid	Low	No	No	No	No	No	No	No	-11.11	yes	0.56	No	2.23
Glycine-Valine	High	No	No	No	No	No	No	No	-9.27	yes	0.55	No	1.81
Alanine-Lysine	High	No	No	No	No	No	No	No	-10.10	Yes	0.55	No	2.55
Aspartic acid-Tyrosine	Low	No	No	No	No	No	No	No	-11.20	Yes	0.56	No	2.71
Glycine-Alanine	Low	No	No	No	No	No	No	No	-9.78	Yes	0.55	No	1.66
Glycine-Glutamine	Low	No	No	No	No	No	No	No	-11.10	Yes	0.56	No	2.13
Proline-Tyrosine	High	No	No	No	No	No	No	No	-9.72	Yes	0.55	No	2.62
Alanine-Phenylalanine	High	No	No	No	No	No	No	No	-4.49	Yes	0.55	No	2.39

Table 2: Molinspiration features with enzyme bioactivity score of peptides molecule.

Peptides	Mi Log P	n Atoms	nON	nOHNH	n violations	Volume	Enzyme Inhibitors Bioactivity Score
Proline-Methionine	-1.83	16	5	3	0	224.94	0.67
Aspartic acid-Cysteine	-4.10	15	7	5	0	194.23	1.06
Glycine-Aspartic acid	-3.98	13	7	5	0	159.74	0.29
Glycine-Valine	-2.38	12	5	4	0	165.89	0.12
Alanine-Lysine	-3.75	15	6	6	1	211.02	0.69
Aspartic acid-Tyrosine	-3.10	21	8	6	1	256.00	0.82
Glycine-Alanine	-4.53	22	9	5	0	277.56	0.02
Glycine-Glutamine	-3.71	14	7	5	0	176.54	0.30
Proline-Tyrosine	-1.30	20	6	4	0	252.87	0.78
Alanine-Phenylalanine	0.56	22	8	4	0	274.11	0.21

Table 3: Pass online bioactivity score & toxicity prediction of peptides molecule.

Peptides	Target for TB	Activity		Possible Adverse and Toxic Effects
		PA	PI	
Proline-Methionine	Peptidyl-dipeptidase Dcp inhibitor	0.802	0.010	Nil
Aspartic acid-Cysteine	Protein- disulfide reductase inhibitor	0.970	0.001	Nil
Glycine-Aspartic acid	Chymosin inhibitor	0.921	0.004	Nil
Glycine-Valine	Peptidyl dipeptidase Dcp inhibitor	0.933	0.003	Nil
Alanine-Lysine	Protein- disulfide reductase inhibitor	0.953	0.002	Nil
Aspartic acid-Tyrosine	Protein- disulfide reductase inhibitor	0.950	0.002	Nil
Glycine-Alanine	Membrane integrity antagonist	0.762	0.055	Ulcer
Glycine-Glutamine	Protein- disulfide reductase inhibitor	0.948	0.002	Nil
Proline-Tyrosine	Peptidyl dipeptidase Dcp inhibitor	0.838	0.007	Nil
Alanine-Phenylalanine	Membrane integrity antagonist	0.940	0.002	Nil

Table 4: Docking Score of dipeptides molecule with ΔG value. As from Table-4, it has been observed that Proline-Methionine was identifies as most promising peptide lead along with estimated energy ΔG value of -8.30 Kcal/mol as compare to Aspartic acid-Cysteine ΔG value of -8.24 Kcal/mol using target oxidoreductase.

Molecule	Target	Full Fitness Kcal/mol	Estimated Energy ΔG Kcal/mol
Proline-Methionine	Oxidoreductase	-2865.41	-8.30
Aspartic acid-Cysteine	Oxidoreductase	-2890.90	-8.24

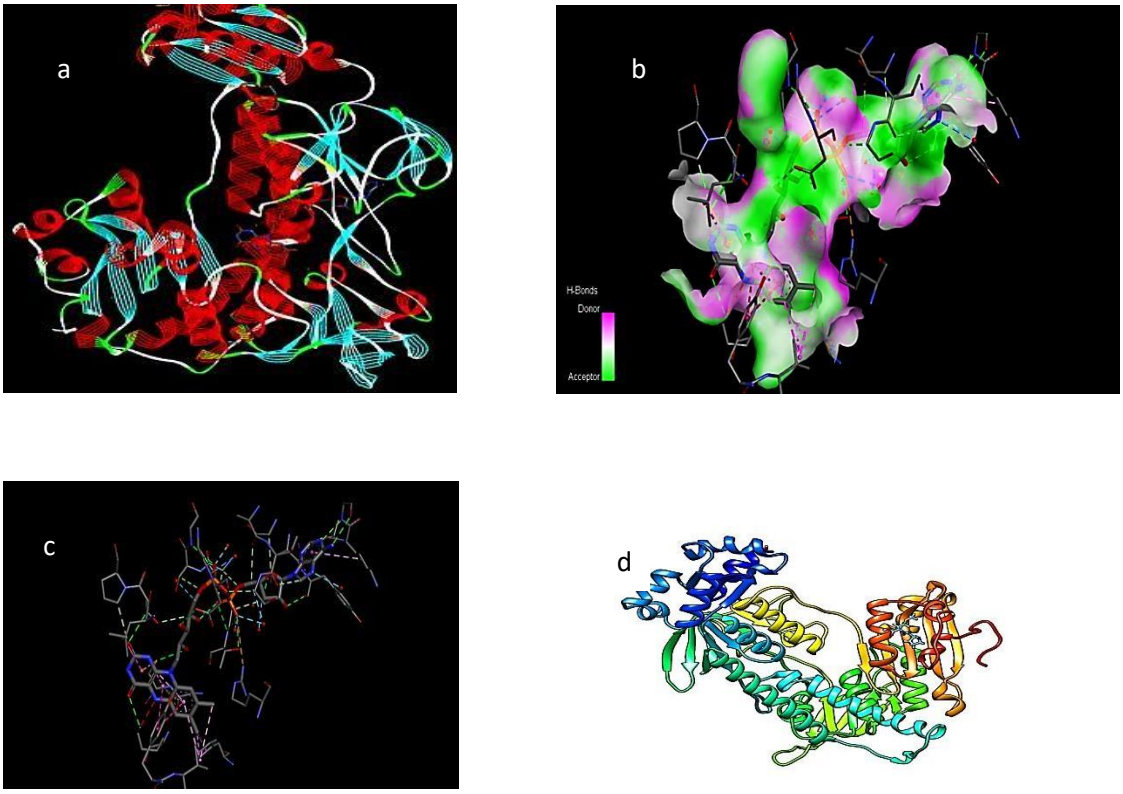

Figure 1: Docking Investigation of dipeptide Proline-Methionine against the target oxidoreductase. (a) 3D Docking of Proline-Methionine with target in ribbon model (b) 3D Pocket view of ligand interaction with enzyme (c) Conventional pharmacophoric distance measurement of H- bond between drug and target using Biovia discovery studio visualizer (d) Computed secondary structure of Proline-Methionine against oxidoreductase target using UCSF chimera model.

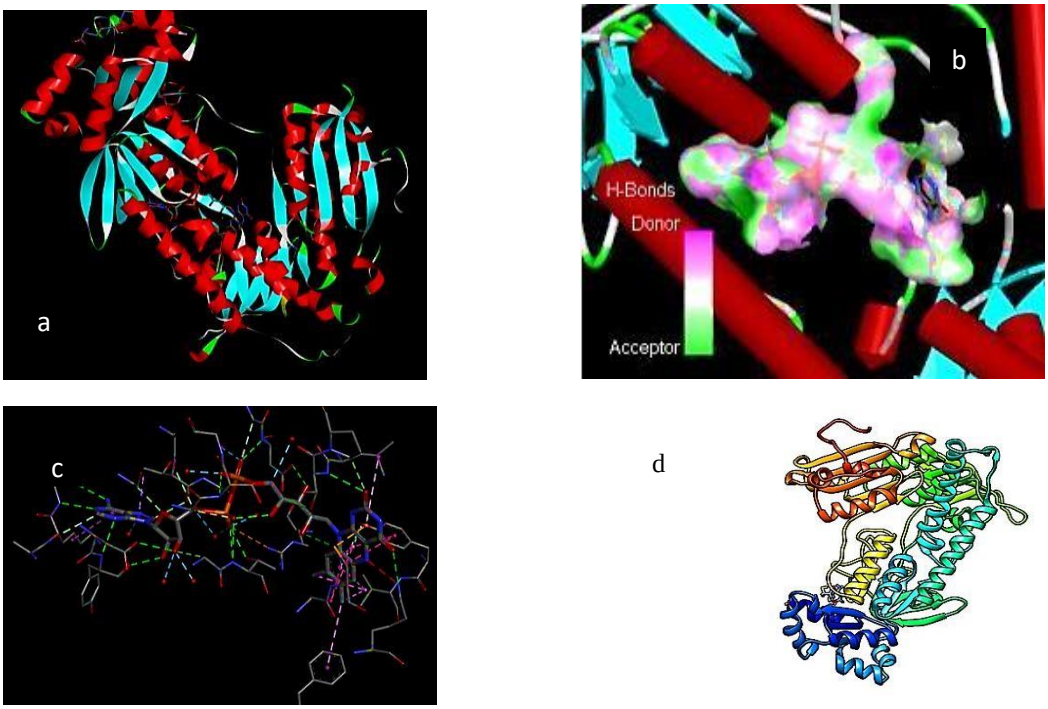

Figure 2: Docking Investigation of dipeptide Aspartic acid-Cysteine against the target oxidoreductase. (a) 3D Docking of Aspartic acid-Cysteine with target in ribbon model (b) 3D Pocket view of ligand interaction with enzyme (c) Conventional pharmacophoric distance measurement of H Bonds between drug and target using Biovia discovery studio visualizer (d) Computed secondary structure of Aspartic acid-Cysteine against oxidoreductase target using UCSFChimera model.

3.2 Synthesis of (Proline-Methionine, Aspartic acid- Cysteine) dipeptides

After the selection of most promising leads through docking strategy is Proline-Methionine, Aspartic acid- Cysteine which has been further synthesize in laboratory using LPPS method, the yield value of the synthesized product shown in Table- 5.

After the synthesis of these dipeptides were purified by fourteen fractions of solvents using column chromatography which observation shown in Table-6 and figure- 3.

Whereas the TLC is used to determine the Rf value of all peptides in Table- 7 using the iodine chamber shown in figure- 3. The melting point of selected peptides is measured and recorded shown in Table- 8.

Table 5: Yield value of synthesized dipeptide

S.NO.	PEPTIDE	YIELD (mg)
1	Proline-Methionine	486
2	Aspartic acid-Cysteine	561

Table 6: Morphological observation of dipeptides purified fraction from column chromatography

Fraction Elute	Solvent system	Ratio (%)	Rf value	
			Proline-Methionine	Aspartic acid-Cysteine
Crude	Dichloromethane	100	-	-
F1	n-Hexane	100	0.125	-
F2	n-Hexane-Ethyl acetate	75:25	0.10	-
F3	n-Hexane-Ethyl acetate	50:50	-	0.85
F4	n-Hexane-Ethyl acetate	25:75	-	-
F5	Ethyl acetate	100	-	0.50
F6	Ethyl acetate-Methanol	75:25	-	-
F7	Ethyl acetate-Methanol	50:50	-	-
F8	Ethyl acetate-Methanol	25:75	0.35	-
F9	Methanol	100	0.70	-
F10	Methanol-Water	80:20	-	0.755
F11	Methanol-Water	60:40	-	-
F12	Methanol-Water	40:60	-	-
F13	Methanol-Water	20:80	-	-
F14	n-Butanol-Acetic acid-Water	40:10:50	-	1.28

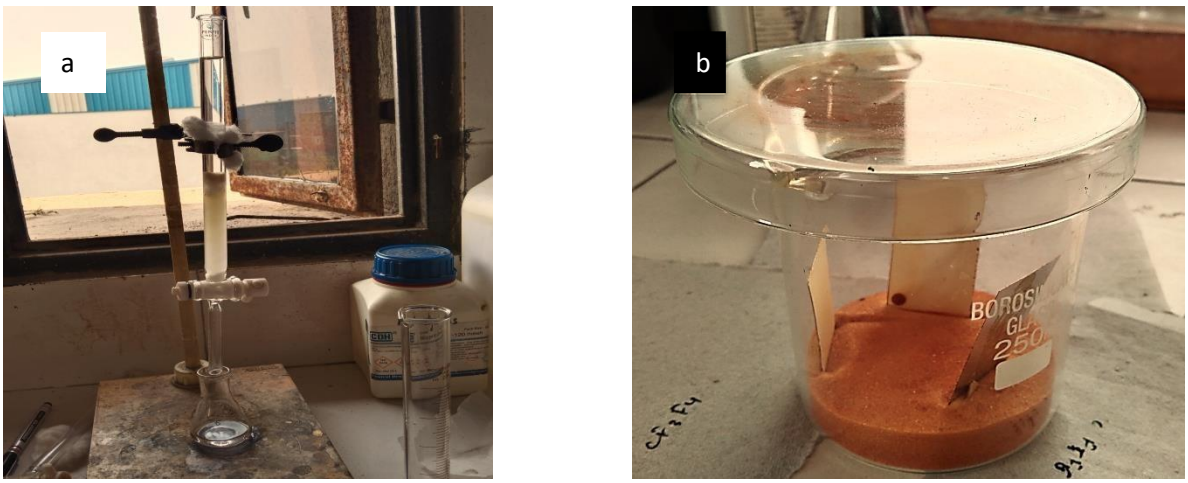

Figure 3: Represent the diagramatic view of (a) column chromatography and (b) Iodine chamber with TLC plates

Table 7: Melting point of dipeptide molecules

Molecule	Melting point (°C)
Proline-Methionine	187
Aspartic acid-Cysteine	197.4

3.3 FT-IR Analysis

The characteristics bond of Proline-Methionine was observed at 2897.08 cm-1 with S-CH_3 functional group, 2698.41 cm^{-1} with (S-) CH_2 functional group, 2897.08 cm^{-1} with C-H functional group, 1492.90 cm^{-1} with N-C=O functional group, 1662.64 cm^{-1} with C=O functional group,1230.58 cm^{-1} with C-N functional group, and 3028.24 cm^{-1} with O-H functional group. The stretching vibration peaks of Proline-Methionine is observed at 1338.60 cm^{-1} with CH functional group, 1230cm^{-1} with NH functional group, and 1400 cm^{-1} with OH functional group.

The characteristics bond of Aspartic acid-Cysteine was observed at 677.01 cm^{-1} with C-S functional group, and 1633.71 cm^{-1} with CO functional group. The stretching vibration peak of Aspartic acid-Cysteine is observed at 2310.72 cm^{-1} with OH functional group, 2349.3$cm^{-1}$with SH functional group, 3140.11 cm^{-1} with NH functional group, 14.4.18 cm^{-1} with CN functional group, and 1498.69 cm^{-1} with CH functional group.

3.4 NMR Analysis

In this research work the experimental shielding range of dipeptides molecules for ^1H NMR is measures as of Proline-Methionine were observed at 1.873 ppm with CH_3 functional group, 2.497 ppm with CH-S functional group,and 4.399 ppm with CONH functional group. Aspartic acid-Cysteine was observed at 2.507 ppm with C-SH functional group, 1.1ppm with CH_3 functional group, 7.359 ppm with CONH functional group, and 3.396 ppm with OH functional group.

3.5 DS-MS Analysis

Interpretation of Mass Spectra of dipeptides sequence
of Proline-Methionine having fragment m/z ratio of
214.48, 215.48 and 207.44 whereas the Aspartic acid-Cysteine having fragment m/z ratio of 218.45, 200.46, 196.39, 149.31 and 117.22.

3.6 In-Vitro Antioxidant activity

In in-vitro antioxidant activity by phosphomolybdenum method was detected at specific concentration of 1µg/ml, 2µg/ml, 4µg/ml, 8µg/ml, 16µg/ml, and 32µg/ml for Proline-Methionine with an IC_{50} value of 7.4µg/ml, which showsthe antioxidant activity greater than Aspartic acid-Cysteine with an IC_{50} value of 8µg/ml as compared with the standard drug riboflavin IC_{50} value of 9.4µg/ml. Value obtained from regression lines with 95% of confidence level. *IC_{50} is defined as the concentration sufficient to obtain 50% of a maximum effect estimate in 100%. All values given are mean of triplicate experiment at S.D (5%)* in Table- 8. The UV- spectrometric graph was made for compound Proline-Methionine which shows best result of IC_{50} and standard drug with their linear regression and correlations coefficient shown in figure 8&9.

Table 8: Phosphomolybdenum activity of dipeptides with standard drug riboflavin.

S.No.	Compound Name	% Inhibition						IC50 (µg/ml)
		1 µg/ml	2 µg/ml	4 µg/ml	8 µg/ml	16 µg/ml	32 µg/ml	
1.	Proline-Methionine	13.07	11.09	11.31	10.34	10.62	100	7.4
2.	Aspartic acid-Cysteine	14.22	21.04	22.87	15.04	13.44	100	8
3.	Riboflavin	54.58	62.30	14.05	17.11	13.17	100	9.4

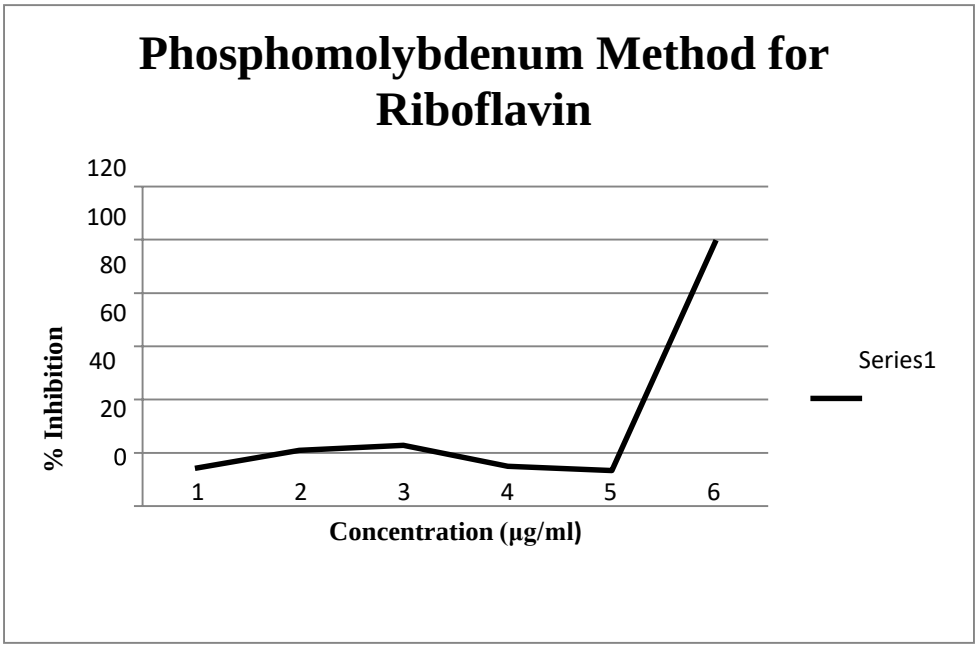

Figure 8: Graphical representation of linearity for the standard drug concentration with their corresponding values

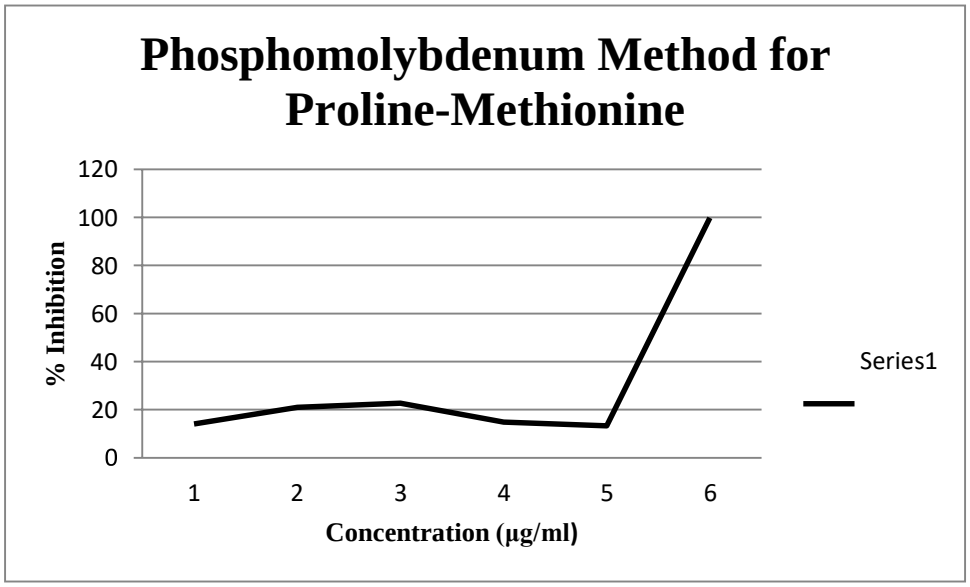

Figure 9: Graphical representation of linearity for the Proline-Methionine concentration with their corresponding values

3.7 Minimum Inhibition Concentration (MIC) of Dipeptides

The minimum inhibitory concentration is the lowest value (μg/ml) of a drug that inhibits the growth of a strain of bacteria. A strain of *Pseudomonas aeroginosa* (ATCC 27853) has an MIC of 4μg/ml for Proline-Methionine and MIC of > 64μg/ml for Aspartic acid-Cysteine along with Levofloxacin taken as standard drug MIC value of 0.5 μg/ml. So, based on MICs, this strain of *Pseudomonas aeroginosa* is more susceptible to Proline-Methionine as compared to Aspartic acid-Cysteine but less susceptible with Levofloxacin as shown in Table- 11.

Table: 11: In-vitro efficacy of dipeptides on Antimicrobial strains demonstrate the minimum inhibitionconcentration (MIC)

Peptides	**Solubility**	**MIC (μg/ml)**	
		***Streptococcus aureus* ATCC 29213**	***Pseudomonas aeroginosa* ATCC 27853**
Proline-Methionine	DMSO	>64	4
Aspartic acid-Cysteine	DMSO	>64	>64
Levofloxacin as standard drug	DMSO	0.125	0.5

3.8 Acute Toxicity Testing of Dipeptides on rodents

This study is to assess the acute toxicity of dipeptides in albino rats. For 14 days, observations and records were made once a day for an additional 4 hours. At the end of the study some behavioral parameters were evaluated like grooming, hyperactivity, convulsions, motor activity, sedation, respiratory arrest, toxicity signs, lacrimation, and urination. Once per day throughout the experiment, rats were weighed and various observations were noted includes mortality, behavior, injuries, and any signs of disease. The rate of mortality has been noted. The oral administration of dipeptides for 14 days may not be toxic, according to the results analysis. Acute toxicity of Proline-Methionine the sign of toxicity, including hypo activity observed at dose of 2000 mg/kg no mortality rate was observed at these three doses 5mg/kg, 50mg/kg, 300mg/kg. The calculated LD_{50} was 1000 mg/kg. In starting dose level, dipeptides were administered at 300 mg/kg dose level revealed the affect during acute toxicity testing studies in albino rats for 72 hours. Grooming was observed, no hyperactivity was observed in rats means remained normal, all rats' remains calm and feel free-stress conditions, no respiratory arrest was observe, no convulsions measure, motor activity not reduced or decreased with zero mortality rate. After increase in dose levels at 2000 mg/kg showed the following effects in rats for 72 hours. After the time period of 48 hours no grooming observed, no sign of hyperactivity, 50% animals seen in (sedative action, respiratory arrest, by raising of head in rats, paddling of the feet, motor activity decreases and 50% animals died after 24 hours) is shown in Table- 9. In Aspartic acid-Cysteine no mortality rate was observed at these three doses 5mg/kg, 50mg/kg, 300mg/kg. The calculated LD_{50} was 1000 mg/kg. At starting dose level, peptides were administered at 300 mg/kg dose level revealing the effect during acute toxicity testing studies in albino rats for 72 hours. Grooming was observed, no hyperactivity was observed in rats means remained normal, all rats remains calm and felt free-stress conditions, no respiratory arrest was observed, no convulsions measure, and motor activity were not reduced or decreased with zero mortality rate. An increase in dose levels at 2000 mg/kg showed the following effects in rats for 72 hours. After the time period of 48 hours, no grooming was observed, no sign of hyperactivity and 50% of animals seen (sedative action, respiratory arrest, rising of the head in rats, paddling of the feet, motor activity decreased and 50% animals died after 24 hours) is shown in Table- 10.

Figure 10: Represent the picture of oral administration of dipeptides in rats.

Table 9: Effect of LD_{50} purified Proline-Methionine dipeptide in rats after oral administration in acute toxicity study.

Dose level mg/kg	**Body weight**			**Onset of sign of toxicity**			**Observation**	**No. of animals undergoing death**	**LD50 cut off**
Proline-Methionine	**Animal body weight t1**	**Animal body weight t2**	**Animal body weight t3**	**Animal 1**	**Animal 2**	**Animal 3**			
5	200	200	200	180	200	150	Sleep/lethargy	None	Dose decreased
50	250	250	250	200	200	200	Sleep/lethargy	None	Dose decreased
300	200	200	200	150	120	120	Sleep/lethargy	None	Dose decreased
2000	300	300	300	250	200	200	Convulsions and respiratory arrest	2	1000 mg/kg

Table 10: Effect of LD_{50} purified Aspartic acid-Cysteine dipeptide in rats after oral administration in acute toxicity study.

Dose level mg/kg	**Body weight**			**Onset of sign of toxicity**			**Observation**	**No. of animals undergoing death**	**LD50 cut off**
Aspartic acid-Cysteine	**Animal body weight t1**	**Animal body weight t2**	**Animal body weight t3**	**Animal 1**	**Animal 2**	**Animal 3**			
5	200	200	200	180	200	150	Sleep/lethargy	None	Dose decreased
50	250	250	250	200	200	200	Sleep/lethargy	None	Dose decreased
300	200	200	200	150	120	120	Sleep/lethargy	None	Dose decreased
2000	250	250	250	200	200	200	Convulsions and respiratory arrest	2	1000 mg/kg

IV Conclusion

The purpose of this research work has been to identify or evaluate the effective strategies to design and develop potent peptide lead as an antimicrobial agent and to prevent the drug-resistant problems which are a global burden nowadays. This study concluded that the scoring function of docking for Proline-Methionine was found to be the most promising lead with a ΔG value of 8.30 Kcal/mol as compared to other selected dipeptides. Another activity in a biological parameter of Proline-Methionine is also measured with the total antioxidant capacity by using the Phosphomolybdenum method which shows a good IC_{50} value of 7.4 μg /ml as compared to standard drug riboflavin having IC_{50} value of 9.4 μg /ml. It is essential to understand the toxicity and safety of dipeptides need to evaluate. Hence, the acute toxicity results showed that the Proline-Methionine has an LD_{50} value of less than 1000 mg/kg and is considered to be slightly toxic. Finally, the *In-Vitro* antimicrobial assay for ESKAPE pathogen proved that Proline-Methionine (dipeptide) was found to be best susceptible lead against the strain of *Pseudomonas aeroginosa* with a MIC value of 4 μg /ml. If we compare the Proline-Methionine dipeptide molecule with test and standard drugs was found to be less resistant and high sensitive than the aspartic acid-cysteine, as well as other marketed bactericidal and broad antimicrobial drugs like amoxicillin, clavulanic acid, piperacillin, tetracycline, nitrofurantoin, chloramphenicol, trimethoprim and sulfonamide against the *gram-ve* bacteria. Lastly, this information may increase the likelihood of treatment success and lead to the appropriate selection of antibacterial drugs to help and combat by slowing the antibiotic resistance.

V Conflict of Interest

The authors affirm that they do not have any competing interests.

VI Acknowledgement

The authors thank gratefully to Prof. (Dr.) K. Nagarajan and Dr. Garima Kapoor for their support. Also, thankful to Dr. (Col.) A. Garg, Director and Dr. Manoj Goel, Joint Director of KIET Group of Institutions for motivation and support.

VII References

- Miller B. R., Gulick A. M. (2016). Structural Biology of Nonribosomal Peptide Synthetases. Nonribosomal Peptide and Polyketide Biosynthesis. Methods in Molecular Biology. Vol. 1401. pp. 3–29. https://doi.org/10.1007/978-1-4939-3375-4_1.
- Boone, K., Camarda, K., Spencer, P. *et al.* Antimicrobial peptide similarity and classificationthrough rough set theory using physicochemical boundaries. BMC Bioinformatics 19, 469 (2018). https://doi.org/10.1186/s12859-018-2514-6.
- McEwen SA, Collignon PJ. Antimicrobial Resistance: a One Health Perspective. Microbiol Spectr. 2018 Mar;6(2). https://doi.org/10.1128/microbiolspec.ARBA-0009-2017.
- H. Mahdi Kareem, “Oxidoreductases: Significance for Humans and Microorganism,” Oxidoreductase, Feb. 2021, https://doi.org/10.5772/intechopen.93961.
- OECD (2002), Test No. 423*:* Acute Oral toxicity - Acute Toxic Class Method, OECD Guidelines for the Testing of Chemicals, Section 4, OECD Publishing, Paris, https://doi.org/10.1787/9789264071001-en.
- Vasileios Bampidis, Giovanna Azimonti, Maria de Lourdes Bastos et al (2020) Safety and efficacy of sodium carboxymethyl cellulose for all animal species. EFSA Journal 2019- 00179. https://doi.org/10.2903/j.efsa.2020.6211.
- Zekun Liu, Junpeng Zhao, Weichen Li, Xinkun Wang, Jingxuan Xu, Jin Xie, Ke Tao, Li Shen, Ran Zhang, "Molecular Docking of Potential Inhibitors for Influenza H7N9", Computational and Mathematical Methods in

Medicine, vol. 2015, Article ID 480764, 8 pages, 2015. https://doi.org/10.1155/2015/480764.

- German Perez, Marcello Mascini, Valentina Lanzone, Manuel Sergi, Michele Del Carlo, Mauro Esposito, Dario Compagnone, "Peptides Trapping Dioxins: A Docking-Based Inverse Screening Approach", Journal of Chemistry, vol. 2013. https://doi.org/10.1155/2013/491827.
- Jiaorong Tan, Jiahong Xu, Guohua Wei, Lijuan Zhang, Long'e Sun, Guangyu Wang, Fei Li, Fengxiang Jiang, "HNF1α Controls Liver Lipid Metabolism and Insulin Resistance via Negatively Regulating the SOCS-3-STAT3 Signaling Pathway", Journal of Diabetes Research, vol.2019. https://doi.org/10.1155/2019/5483946.
- Tuba Özdemir Öge, "FT-IR, Laser-Raman, UV-Vis, and NMR Spectroscopic Studies of Antidiabetic Molecule Nateglinide". Journal of Spectroscopy, vol. 2018. https://doi.org/10.1155/2018/8573014.
- Naheed N. Kaderbhai, David I. Broadhurst, David I. Ellis, Royston Goodacre, Douglas B. Kell, "Functional Genomics via Metabolic Footprinting: Monitoring Metabolite Secretion by *Escherichia coli* Tryptophan Metabolism Mutants Using FT–IR and Direct Injection Electrospray Mass Spectrometry", International Journal of Genomics, vol. 4. https://doi.org/10.1002/cfg.302.
- Acácio Rodrigues, C. Pina Vaz, A. Freitas da Fonseca, J. Martinez de-Oliveira, "Evaluating the Concentration of a *Candida albicans* Suspension", Infectious Diseases in Obstetrics and Gynecology, vol. 1,1993. https://doi.org/10.1155/S1064744993000304.
- Sharma A, Kumar A, de la Torre BG, Albericio F. Liquid-Phase Peptide Synthesis (LPPS): AThird Wave for the Preparation of Peptides. Chem Rev. 2022 Aug 24;122(16):13516-13546. https://doi.org/10.1021/acs.chemrev.2c00132.
- Bragina ME, Daina A, Perez MAS, Michielin O, Zoete V. The SwissSimilarity 2021 Web Tool: Novel Chemical Libraries and Additional Methods for an Enhanced Ligand-Based Virtual Screening Experience. Int. J. Mol. Sci., 2022 Jan, 23(2). https://doi.org/10.3390/ijms23020811.
- Kandasamy Nagarajan, Anjali Saini, Tanya Goyal, Parul Grover, Garima Kapoor, Roma Ghai, Richa Goel, Vinay Kumar, Vidhu Saxena & Dolly Chauhan (2022), Computer-aided Drug Design of New Tripeptides as Inhibitors of Mycobacterium Tuberculosis Protein Tyrosine Phosphatase, YMER J. https://doi.org/10.37896/YMER21.11/40.
- Prieto P, Pineda M, Aguilar M. Spectrophotometric quantitation of antioxidant capacity through the formation of a phosphomolybdenum complex: specific application to the determination of vitamin E. Anal Biochem. 1999 May 1;269(2):337-41. https://doi.org/10.1006/abio.1999.4019.
- Anjali Saini, Kandasamy Nagarajan, Garima Kapoor, Parul Grover, Khushi Shah & Hariom Tomar (2022), Evaluation of In-vitro antioxidant potency of smaller chain peptides using DPPH radial scavenging activity, Hydrogen peroxide (H2O2) scavenging activity, and Reducing power method (RP), YMER J. https://doi.org/10.37896/YMER21.11/H8.
- Hadda, Taibi B., et al. "Petra/Osiris/Molinspiration and Molecular Docking Analyses of 3- Hydroxy-Indolin-2-one Derivatives as Potential Antiviral Agents." Current Computer-Aided Drug Design 17.1 (2021). https://doi.org/10.2174/1573409916666191226110029.
- Filimonov D.A., Lagunin A.A., Gloriozova T.A., Rudik A.V., Druzhilovskii D.S., Pogodin P.V., Poroikov V.V. (2014). Prediction of the biological activity spectra of organic compounds using the PASS online web resource. Chemistry of Heterocyclic Compounds, 50 (3), 444-457.
- Anonymous et al 2003, Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID), VOLUME 9, ISSUE 8, PIX-XV, AUGUST 01, 2003. https://doi.org/10.1046/j.1469- 0691.2003.00790.x.
- Lipinski CA (December 2004). "Lead- and drug-like compounds: the rule-of-five revolution". Drug Discovery Today: Technologies 1 (4**)**: 337â€“34