Research Journal of Pharmaceutical Sciences ISSN 2319 – 555X
Vol. **10(1),** 1-7, June **(2021)** *Res. J. Pharmaceutical Sci.*

Invitro studies for immuno stimulant activity of Cuttle fish ink

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Available online at: www.isca.in, www.isca.me
Received 14th December 2020, revised 29th April 2021, accepted 25th May 2021

Abstract

In the Marine organisms were isolated the 10,000 of new compounds were being discovered every year. Huge innovations were obtained from the waste, by product of the marine sources to discover the new compounds to the consumers. In the 21st century, people are interested in healthy eating that shift towards the potential health benefits beyond basic nutrition. Cuttle Fish Ink was evaluated for Immunostimulant activity by using invitro studies. Phagocytic index and cell count in Immunomodulatory assay, molecular docking studies of the compounds such as Phenyl thiazole, Boldenone using Insilico docking analysis. Our results showed the Cuttle Fish Ink possess Immunostimulant activity and molecular docking score produces with appreciable and better therapeutic effect in the ADME properties.

Keywords: Cuttle Fish Ink, Immunomodulatory, Molecular docking, *Insilico* docking, Phagocytic index.

Introduction

In recent years, great attention towards the potential pharmacological utilization of bioactive natural products[1]. Mollusca is the 2nd largest phylum in the marine environment around 1,00,000 species found in all over the world[2]. Huge innovations were obtained from the waste by product of the marine sources to discover the new compounds to consumers[3].

The ink sac with its ink gland produces a black ink containing melanin, the funnel shaped organ containing mucus producing gland. Cuttlefish is mainly used in homeopathic medicine. It is also used namely such as food products, antiseptic, antibacterial and antitumor agent[4].

Antibody molecule consists of two light and two heavy chains composed of different domains i. Fab fragment, ii. Fc fragment. Fab fragment serves as the antigen binding site. Fc fragment is relatively constant and determines the effect or function of the antibody[5]. Many bacterial products are PAMPs, they strongly stimulate inflammation by triggering cytokine production in APCs. These in turn, stimulate the adaptive immunity and overall increase leukocytes number by boosting hematopoiesis[6]. Immunization is the administration of antigen to the host in order to induce antibody production. Vaccines are used for active immunization, they impart active immunity, which takes some time to develop and therefore used prophylactically. The antibodies so developed destroy the specific microorganism when it enters the body[7]. An immune response required the both innate immunity and the more powerful flexible acquired immunity[8].

Materials and methods

Extraction: The fresh Cuttle fish was collected directly from the Muthupettai region of Nagapattinam and dissected the fish collected the Ink Sac and transferred immediately in ice box to extract the Ink. The Ink was dissolved with equal volume of Phosphate Buffer Saline (pH-7) immediately ultrasonicated the extract and then the extract was centrifuged at 5000rpm for 10 minutes, and the supernatant liquid was stored at -20°C until use it should freeze[9].

Preparation of Phosphate Buffer Saline (PBS): 2.38g – Disodium Hydrogen Phosphate, 0.19g – Potassium Hydrogen Phosphate, 0.8g – Sodium Chloride weighed and mixed with distilled water to produce 1000ml, pH adjusted at 7.

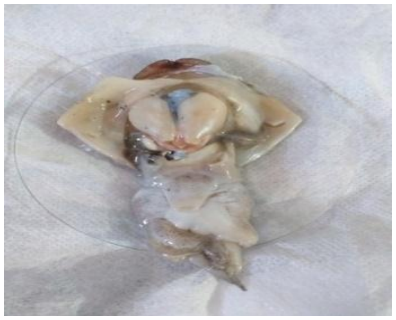


Figure-1: Dissection of Cuttle fish.

Figure-2: Cuttle Fish Ink sac.

Invitro Immunostimulant assay: By finger prick method the blood was collected and placed to the blood to the glass slide and spread upto 1.5×1.5cm. Blood was allowed to clot at 37°C for 25 min. By using sterile normal saline the blood clot was removed from the slide. The polymorph nuclear leukocytes (PMN's) were found on the glass surface, the rest of the blood components are washed away. Duplicate slides were prepared and used for the Cuttle fish ink extract.

0.1ml of Cuttle fish ink extract was flooded over the slides, the slides were incubated at 37°C for 15 min followed by the addition of 100µl of *C. albicans* cell suspension. The slides were incubated at 37°C for 60 min. After incubation period, washed the film for twice with normal saline. By using the methanol to fixed the film for 5 min. Diluted Giemsa stain was flooded over the film for 25 min. The excess stain was removed using PBS and air dried. The slides were observed under the oil immersion (×100) objective. In microscopically to determined the number of *Candida cells* phagocytosed to detect the morphological criteria. This number was taken as the phagocyte index (PI) and was compared with the PI of the control. Immuno stimulation (%) was calculated by using the following Equation[10,11].

$$\text{Stimulation (\%)} = \frac{\text{PI (test) –PI (control)}}{\text{PI (control)}} \times 100$$

Molecular docking *for Immunostimulant:* Tool: autodock vina 1.2 was used for docking analysis[12-14].

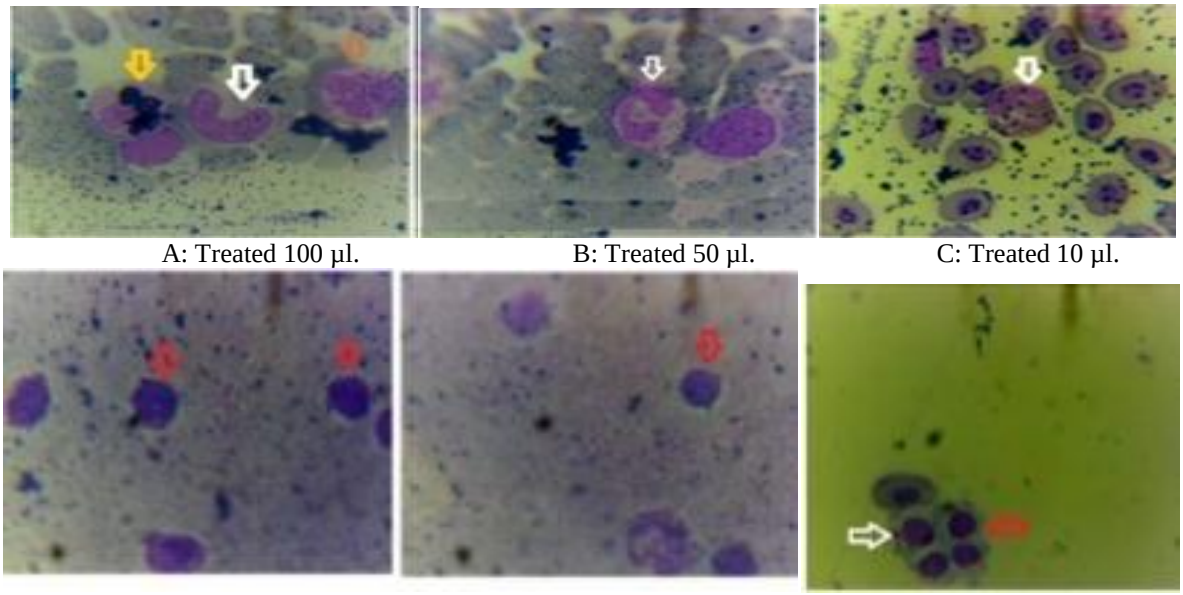
Receptor protein: In the RCSB Brookhaven Protein Data Bank [PDB entry code 1M48 and 1PW6] were retrieved the Crystal structures of the IL-2 and used for docking studies. The structure 1M48 with was used for docking. All water molecules, except HOH357, and ligand were removed and hydrogen were added to the original protein data bank file.

Ligand: The ligands from squid ink (4-phenyl thiazole, Boldenone) were constructed by using standard bond lengths and angles from the SYBYL 7.3 fragment library. Geometry optimizations were performed and Gasteiger–Huckel charges were used for the ligands.

DOCKING: The entire docking study was determined by Autodock vina equipped with UCSF chimera and the ligands were focused on the active site of the viral protein receptors to perform site-specific docking to procure potential inhibitors. The residues of the binding sites were manually determined for the possible flipped orientation, protonation, and tautomeric states with Pymol 1.3 (Delano Scientific, San Carlos, USA) side-chain wizard script. The binding sites were defined as all the amino acid residues encompassed within a 10.0 a radius sphere centered on catalytic. The successful ligands obtained from screening analysis were further investigated for ADMET (absorption, distribution, metabolism, excretion and toxicity) properties[15].

Results and discussion

Immunomodulatory *assay: Invitro* evaluation of phagocytosis, extract of Cuttle fish Ink at 10µl, 50µl and 100µl showed, morphological changes in the granulocytes given in (Figure-3). Maximum phagocytic activity was recorded as 71% at 100µl followed by 28% at 50µl. In this study dose-dependent shift on neutrophils in *invitro* phagocytosis was noted. At 10µl no stimulation was recorded but at the high concentration elevation of the phagocytic index.



A: Treated 100 µl. B: Treated 50 µl. C: Treated 10 µl.

D: Untreated Phagocytic.

Figure-3: Immunostimulatory effect of Cuttle fish ink.

Immunostimulation (%) was calculated by using the equation

$$\text{Stimulation (\%)} = \frac{\text{PI (test) –PI (control)}}{\text{PI (control)}} \times 100$$

Table-1: Immunostimulation (%).

Sample	PI	Stimulation (%)
Control	28	
Test 10 µL	28	0
Test 50 µL	36	28
Test 100 µL	48	71

Molecular Docking for *Immunostimulant:* ADME properties of GCMS–NIST matched compounds: Pharmacokinetics, bioavailability, drug-likeness of Cuttle fish Ink in studied by ADME properties. Out of 16 different compound from cuttle fish ink GCMS analysis 4 compounds are poorly soluble, two are moderately soluble, five are soluble and 4 are highly soluble in nature with permitted range of Log S (not exceed +6).The prediction of bioavailability revealed that same bioavailability scores were obtained for 13 small molecules (0.55) and 0.85 for three molecules. All the 16 obeyed Lipinski's 'rule of five' further BBB absorption reveals 9 compounds were found to be positive result on BBB barrier. Based on pharmacokinetics **Phenyl thiazole and Boldenone were selected for docking**[16].

Sequence similarity analysis of selected target receptor: Pair wise Sequence Alignment by EMBOSS Needle algorithm of receptor 1M48 (001) and 1PW6 (002) is given below (Figure-4), Out of 133 residues similarity percentage was 99.2% and the fealty gap was 0.8% was given in (Figure-5)[17,18].

```
EMBOSS_001         1 -PTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA     49
                     |||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002         1 APTSSSTKKTQLQLEHLLLDLQMILNGINNYKNPKLTRMLTFKFYMPKKA     50

EMBOSS_001        50 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSE     99
                     ||||||||||||||||||||||||||||||||||||||||||||||||||||||
EMBOSS_002        51 TELKHLQCLEEELKPLEEVLNLAQSKNFHLRPRDLISNINVIVLELKGSE    100

EMBOSS_001       100 TTFMCEYADETATIVEFLNRWITFCQSIISTLT    132
                     |||||||||||||||||||||||||||||||||
EMBOSS_002       101 TTFMCEYADETATIVEFLNRWITFCQSIISTLT    133
```

Figure-4: Similarity analysis of selected target receptor.

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Aligned_sequences: 2
1: EMBOSS_001
2: EMBOSS_002
Matrix: EBLOSUM62
Gap_penalty: 10.0
Extend_penalty: 0.5

Length: 133
Identity:     132/133 (99.2%)
Similarity:   132/133 (99.2%)
Gaps:           1/133 ( 0.8%)
Score: 676.0
```

Figure-5: Residues Similarity percentage.

Table-2: ADME properties of NIST matched compounds.

Comp. Name	Mol.wgt (g/mol)	Number of Hydrogen		Water solubility	GI absorption	Bioavailability score	BBB absorption	Lipinski	Log P	Log S
		Accept	Donor							
Docosanoic acid	340.58	2	1	Poorly soluble	Low	0.85	No	Yes,4.15	9.19	7.05
Hexadecanoic acid	256.42	2	1	Moderate soluble	High	0.85	Yes	Yes,4.15	7.23	5.80
1,2-Benzene dicarboxylic acid	164.11	4	0	Very soluble	High	0.85	Yes	Yes,0	1.22	1.73
Phthaic acid, 6 ethyl oct-3-yl phenyl ester	373.42	4	0	Poorly soluble	High	0.55	Yes	Yes,4.15	6.92	6.14
Dimethyl 3-(2-thienyl)-4,5-isoxazoledi carboxy late	268.27	6	0	soluble	High	0.55	No	Yes, 0	2.03	3.45
2-(2-methyl piperidino) ethyl p-chloro benzoate	281.78	3	0	Moderate soluble	High	0.55	Yes	Yes, 0	3.44	3.34
Cyclononasiloxaneoctadeca methyl	667.39	9	0	Poorly soluble	Low	0.55	N0	Yes, 500	5.56	6.21
Glutaric acid, di(2-methoxy phenyl) ester	344.36	6	0	Soluble	High	0.55	Yes	Yes, 0	3.30	4.53
Fumaric acid, 3-fluorophenyl hexadecyl ester	434.58	5	0	Poorly soluble	Low	0.55	No	Yes,4.15	8.49	7.15
Phenyl thiazole	161.22	1	0	Soluble	High	0.55	Yes	Yes, 0	2.72	2.58
1-(4-Pyrimidinyl) ethanone	122.12	3	0	Very soluble	High	0.55	Yes	Yes,0	0.01	0.09
Boldenone	286.41	2	1	Soluble	High	0.55	Yes	Yes,0	3.08	4.04
6H Purin-6-one, 2 amino-1,7 dihydro	169.14	4	4	Very soluble	High	0.55	No	Yes, 0	0.66	0.33
2,2-dimethyl 2 (2,3,5,6-tetramethyl) ethanol	206.32	1	1	Soluble	High	0.55	Yes	Yes, 0	4.39	3.73
Dicyclohexylphosphinic acid	230.28	2	1	Soluble	High	0.55	Yes	Yes,0	3.48	2.23
6 hydroxy 5- (methyimino) methyl-2,4(1H,3H) pyrimidinedione	169.14	4	3	Very soluble	High	0.55	No	Yes,0	1.64	1.64

***In silico* docking of 4-phenylthiazole with 1PW6-L:** The co-crystallized ligands, namely 1PW6-L, is docked into active site by multipose docking with 4-phenylthiazole and Boldenone. Crystal Structure of Human IL-2 1PW6-L Complexed with (R)-N-[2-[1-(Aminoiminomethyl)-3-piperidinyl]-1-oxoethyl]-4-(phenylethynyl)-L-phenyl alanine methyl ester was given in (fig.6) and ligand is given in (Figure- 7).

Interaction with 4-phenylthiazole (Figure-8) formed 3 major hydrogen bonds with ARG 81 residues at pose v13, v14 and V27 with the distance of 3.287, 2.585 and 3.189A° (Table-3). V13 and V27 Arginine Residue interacted with S1 atom of 4-phenylthiazole whereas V14 residues form bonding with N1 of ligand. All the three poses showed same amino acid with docking score of -5.

Table-3: In silico docking of 4-phenylthiazole with 1PW6-L.

Pose	Docking score	Energy	Amino acid	H bond Distance A
V13/2	-5.352062	4.78002	ARG81-LIG S1	3.287
V14-1	-5.9690866	3.22708	ARG81-LIG N1	2.585
V27/2	-5.295699	6.08042	ARG81-LIG S1	3.189

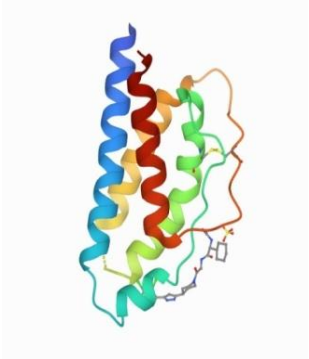

Figure-6: Crystal structure of 1PW6-L.

Figure-7: Ligand.

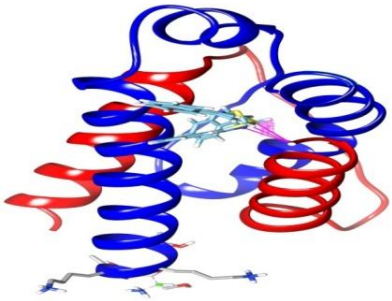

Figure-8: Interaction of 4-phenylthiazole.

***In silico* docking of 4-phenylthiazole with 1M48-L:** The co-crystallized ligands, namely 1M48, is docked into active site by multipose docking with 4-phenylthiazole and Boldenone. Crystal Structure of 1M48 Complexed with (R)-N-[2-[1-(Aminoiminomethyl) – 3 - piperidinyl] – 1 - oxoethyl] - 4-(phenylethynyl)-L-phenylalanine methyl ester was given in (Figure-9) and Interaction of ligand with 4-phenylthiazole (Figure-10) formed 2 major hydrogen bonds with ARG 81 residues at pose V28/1 and V31/1 with the distance of 2.407 and 2.735A (Table-4). V28 Arginine Residue interacted with N1 atom of 4-phenylthiazole whereas V31 residues form bonding with S1 of ligand. All the two poses showed same amino acid with docking score of -5 and -4.

Table-4: In silico docking of 4-phenylthiazole with 1M48-L.

Pose	Docking score	Energy:	Amino acid	H bond Distance A
V28/1	-5.4773726	3.78236	ARG81-LIG N1	2.407
31/1	-4.8410544	4.79036	ARG81-LIG S1	2.735

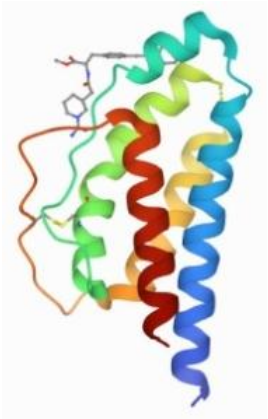

Figure-9: Crystal structure of 1M48-L.

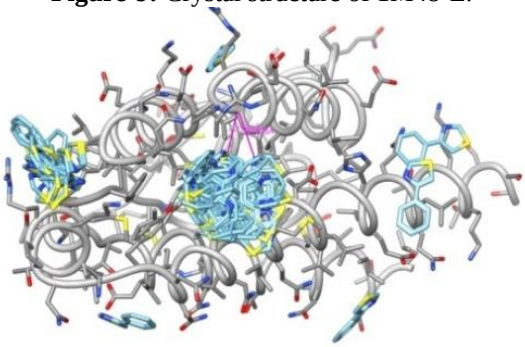

Figure-10: Interaction of ligand with 1M48-L.

***In silico* docking of Boldenone with 1PW6-L:**The structure of ligand given in (Figure-11) and predicted binding pose between Boldenone and 1PW6-L was in good and found 11 stable hydrogen bond possibilities (Figure-12). Table-5 shows number of hydrogen bond, distance and residues take part in interaction. Boldenone interacted with ARG81, LEU70 and GLN74. The maximum docking score was -7.02649 formed by LEU70 with 2.617A° long hydrogen bond followed by ARG81 have score of -6.8500466 and 2.714A° hydrogen bond. The docking score of GLN is -5.45621735 and the distance of H bond is 2.210A°.

***In silico* docking of Boldenone with 1M48-L:** The interaction of ligand given in (Figure-13) and predicted binding pose between Boldenone and 1M48 was in good and found 6 stable hydrogen bond possibilities. Table-6 shows number of hydrogen bond distance and residues take part in interaction. Boldenone interacted with ARG81 and ARG81H. The maximum docking score was -6.9702897 formed by ARG81H with 2.362A and it also have a long hydrogen bond. The docking score of ARG81 is -6.943564 and the distance of H bond is 1.922A.

Table-5: In silico docking of boldenone with 1PW6-L.

Pose	Docking score	Energy:	Amino acid	H bond Distance A
V1/1	-5.984672	17.9627	ARG81H-LIG H47	2.255
V1/1	-5.7518616	19.6981	ARG81O-LIG S1	2.234
V1/1	-6.9709845	25.7595	ARG81H-LIG O	2.038
V1/1	-6.705144	27.8	ARG81H-LIG O	2.211
V2/2	-7.02649	11.2959	LEU70 LIG H47	2.617
V3/1	-6.8500466	10.3027	ARG81O-LIG H47	2.714
V4/1	-6.015827	21.1666	ARG81O-LIG O25	2.350
V16/1	-5.6501822	22.889	ARG81H-LIG O14	2.649
V22/1	-5.7811737	21.2966	LEU70-LIG H47	2.389
V24/1	-5.45621735	22.4746	GLN 74-LIG H47	2.210

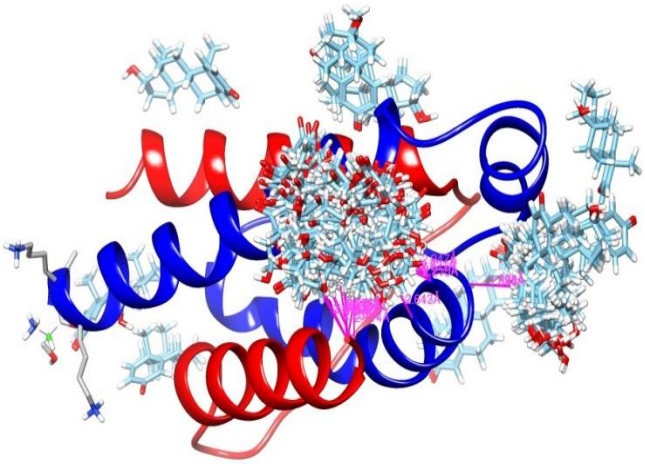

Figure-11: Boldenone ligand.

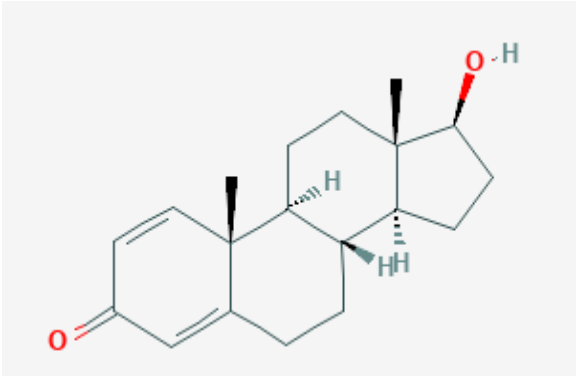

Figure-12: Interaction of ligand with 1PW6-L IL receptor.

Table-6: In silico docking of boldeone with 1M48-L.

Pose	Docking score	Energy	Amino acid	H bond Distance A
V0/1	-6.943564	13.0824	ARG81-LIG O14	1.922
V0/2	-6.8775163	15.1162	ARG81H-LIGO14	2203
V0/2	-6.822891	15.613	ARG81H-LIGO14	1.980
V0/1	-6.1994705	14.9614	ARG81H-LIGO14	1.965
V2/1	-6.9702897	13.9473	ARG81H-LIGO14	2.362
V35/1	-5.4658237	17.5381	ARG81H-LIGO25	2.346

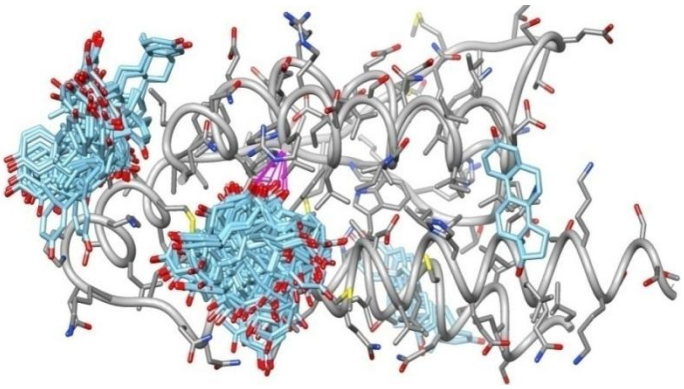

Figure-13: Interaction of boldenone with 1M48-L.

Conclusion

From the results it was concluded that the Cuttle Fish Ink have a Immunostimulant activity, it showed that the Phagocytosis of cells were counted and the morphological changes in the granulocytes was recorded as it possess the better effects, and the Molecular docking of *Insilico* models the compounds are Phenyl thiazole and Boldenone interaction with the Interleukin receptors produced more effect, docking score -5 to -7 hence its produces the better therapeutic effect of Immunostimulant activity.

Acknowledgement

We owe our sincere thanks to the Honorable Chairperson Dr. K. Veeramani, Periyar Maniammai Institute of Science and Technology (PMIST), India and the Principal Dr. R. Senthamarai, Periyar College of Pharmaceutical Sciences, Tiruchirappalli, India for providing necessary facilities and infrastructure to do the research and also I convey my gratefulness to Dr. A. Raja, Executive Director, Helixium Research Academy, Tiruchirappalli, India for his valuable guidance and helping in the docking studies. I express my earnest thanks to The Tamilnadu Pharmaceutical Sciences Welfare Trust, Chennai for their corpus fund to my project which encouraged me in my research work.

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