Int. J. Drug Res. Tech. 2015, Vol. 5 (1), 35-46 ISSN 2277 - 1506

International Journal of Drug Research and Technology

Available online at http://www.ijdrt.com

Original Research Paper

ANTIMYCOBACTERIAL ACTIVITY OF *KAPPAPHYCUS ALVAREZII* AGAINST *MYCOBACTERIUM TUBERCULOSIS* AND *IN SILICO* MOLECULAR DOCKING OF KAPPA-CARRAGEENAN AGAINST InhA ENZYME

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ABSTRACT

In the present study, the antimycobacterial activity of *Kappaphycus alvarezii* against $H_{37}R_V$ and MDR clinical isolate strains of *Mycobacterium tuberculosis* was carried out using three different solvent extracts (acetone, chloroform and ethanol) at two different concentrations (100 µg/ml and 500 µg/ml) by Luciferase Reporter Phage assay. The analysis depicted that 500 µg/ml of acetone and chloroform extracts have a significant antimycobacterial activity against $H_{37}R_V$ strain of *Mycobacterium tuberculosis*. On the other hand, all the three extracts at both 100 µg/ml and 500 µg/ml have antimycobacterial activity against the clinical isolate strain of *Mycobacterium tuberculosis*. The *in silico* docking of kappa-carrageenan against the InhA enzyme were done by AutoDock Software and Accelrys Discovery Studio Visualization Tool and the docking studies found out that kappa-carrageenan formed one hydrogen bond with the docking score of -11.5 with InhA enzyme. This reveals that kappa-carrageenan has inhibitory activity against InhA enzyme, thus controlling the activity of *Mycobacterium tuberculosis.*

Keywords: *Kappaphycus alvarezii*, *Mycobacterium tuberculosis*, InhA enzyme, AutoDock.

INTRODUCTION

Tuberculosis abbreviated as TB for *tubercle bacillus* is one of the most common and deadly infectious disease caused by *Mycobacteria*, mainly *Mycobacterium tuberculosis*.[1] According to WHO (2005)[2] report, TB remains a public health issue in many parts of the world. TB is the leading cause of death in the world with prevalence of 1/3rd the world's population, an incidence of 9 million cases each year, and 5% of the cases are bacteria resistant to anti-TB drugs.[3] *Mycobacterium tuberculosis* has two features that render it the deadliest infectious disease to date, its high infectivity (virulence) and its ability to enter latency for subsequent reactivation, a phenomenon that leads to a deadly synergy with AIDS.[4,5,6] According to the World Health Organization, 1.6 million people died of TB in 2005. The disease is a bigger killer than malaria and HIV/AIDS combined and takes the lives of more women each year than all combined caused of maternal mortality.[7] TB caused 1.3 million deaths among HIV-Negative people and 0.38 million deaths among HIV-Positive people in 2009[8] that the good news is that tuberculosis is a disease that we know a lot about and can cure. If it is treated properly and the patient takes all of TB medicines, then he can be cured and left untreated, TB in the lungs or anywhere else in the body can kill.[7] Current treatment of TB is based on drugs that are more than 40 years old. Despite a demonstrated high efficacy in clinical trials[9], standardized short course chemotherapy (SCC) of active drug-susceptible TB requires direct supervision to assure good adherence and prevent drug resistance.[10] Drugs that are active against resistant forms of TB are less potent more toxic and need to be taken for a long time (≥18 months). The recent emergence of virtually

untreatable extensively drug-resistant TB (XDR-TB) poses a new threat to TB control worldwide.[4] It has been reported that 9-month regimen of isoniazid (INH) is the preferred option for treatment of LTBI in all patients. The 4-month rifampin regimen (six months in children) is an acceptable alternative, especially if there are adverse reactions or resistance to INH, but not rifampin, or the individual is not going to be available for more than 4 to 6 months and is thus unlikely to complete a 9-month INH regimen.[12] Seaweeds are admirable source of medicine. To date, there are quite a lot of reports on antibacterial activity of solvent extracts from marine algae. However, there are very few reports pertaining to antifungal activity of crude solvent extracts from the seaweeds representing *Phaeophyceae* and *Rhodophyceae*.[13] Many workers have also reported antimicrobial activities of marine algae.[14,15,16] Red algae contain various inorganic and organic compounds, that are beneficial for human health[17] because of their high nutritional value and their curative properties for many diseases (TB, arthritis, colds, influenza, worm infestation and tumors).[18] In recent years, much attention has been focused on polysaccharides isolated from natural sources. During the last decade, numerous bioactive polysaccharides with interesting functional properties have been discovered from seaweeds. Several algal species belonging to *Phaeophyta, Rhodophyta* and *Chlorophyta* divisions have been recognized as crucial sources of sulfated polysaccharides (SP). These SP constitute an important ingredient of cell walls and get harvested by suitable extraction or precipitation method, followed by purification, characterization and biological studies.[19] Carrageenans are sulfated linear polysaccharides extracted from certain red seaweed of the *Rhodophyceae* class. They have been extensively used in the food industry as thickening, gelling agent and more recently used in the food industry as excepient in pill and tablets.[20] Kappa-carrageenan which can from strong gel is highly valued in dairy application. A good source of kappa-carrageenan is *Eucheuma cottonii*, which is mainly harvested in the Philippines and Indonesia. The yield and physical properties of carrageenan such as gel strength, gelling and melting temperature as well as chemical properties, determine its values to the industry.[21] In the food industry, structural isomeric forms of kappa and iota-carrageenans are wieldy used as gelling, stabilizing and viscosity-building agents (thickeners) for the preparation of several products, including chocolate-flavored milk, frozen desserts, soymilk, cottage cheese dressings and some diet products.[22] Red seaweed galactan sulfates are linear polysaccharides with alternating 3-linked β-D-galactopyranose units and 4-linked 3,6-anhydro-α-galactopyranse or α-galactopyranose units, having different positions and degrees of sulfation. Other subsitituents, as methyl ethers, pyruvic acid ketals and single stubs of β-D-xylopyranose and/or other monosaccharides are sometimes present. They have been divided in carrageenans, when these 4-linked residues (B-units) are on the D-configuration, and in agarans, when these residues belong to the L-series. Thus, two diastereomeric polysaccharide groups are defined, and the seaweeds that biosynthesize these polysaccharides are called carrageenophytes and agarophytes, respectively.[23] A survey of literature revealed that work on the pharmaceutical application of *Kappaphycus alvarezii* is less. In view of this it was thought that it will be worthwhile to explore the medicinal properties of *Kappaphycus alvarezii* and its role in combatting TB causing bacteria which will pave a new way for treating TB. Moreover the mechanism of *Kappaphycus alvarezii* in controlling the activity of the disease pathogen will also be done by molecular docking of kappa-carrageenan, the active component present in *Kappaphycus alvarezii* against enoyl reductase (InhA) enzyme present in $H_{37}R_V$ and MDR clinical isolate of *Mycobacterium tuberculosis* and predict the mode of action of the drug.

MATERIALS AND METHODS

Kappaphycus alvarezii was collected during the month of April 2011 from the sea coast of the Parangipettai, Cudallore Dist, Tamil Nadu, India in the form of live sample and transported to the

laboratory in polythene bags. The sample was processed as per the method of Andu *et al.* (2000).[24] Sample was cleaned and epiphytes and necrotic parts were removed. Sample was then rinsed with distilled water to remove any associated debris. Sample was then kept under sunshade for 10 days for drying. After drying, it was ground thoroughly to powder form. This powder was stored in a refrigerator in an airtight container until further use. The sample was extracted using three different solvents, *viz*., acetone, chloroform and ethanol. The antimycobacterial work was carried out at Tuberculosis Research Centre, (TRC) Chetpet, Chennai. For this, two strains of *Mycobacterium tuberculosis viz*., $H_{37}R_V$ (drug sensitive reference strain) and MDR strain (clinical isolate strain) were used. Antimycobacterial activity of *Kappaphycus alvarezii* was evaluated using Luciferase Reporter Phage (LRP) assay using the method of Jacobs *et al*. (1993).[25] The significance of the values between various extracts and concentrations were analyzed using 'Two way ANOVA'.[26] *In silico* molecular docking of kappa-carrageenan was done against enoyl reductase (InhA) enzyme present in both strains of *Mycobacterium tuberculosis*. For molecular docking studies, first the 2-D and 3-D structure of kappa-carrageenan was downloaded from ChemSketch database. Likewise the compound summary of kappa-carrageenan was obtained from PubChem database. Similarly, InhA enzyme sequence was downloaded from SWISSPROT database in FASTA format. Domain analysis of InhA enzyme was done by using PFAM database. The 3-D crystalline structure of InhA enzyme was downloaded from PDB database. The ligand binding site was predicted using Q-SiteFinder database. For docking kappa-carrageenan with InhA enzyme, first the grid was generated by AutoDock Tools software. The docking scores were set in the AutoDock Tools and were finally confirmed for docking of the kappa-carrageenan ligand against InhA enzyme. Once the scores were set and final confirmation was given the AutoDock software docks the ligand against the InhA enzyme. The molecular visualization of the dock was done by Accelrys Discovery Studio visualizer 2.5 software.

RESULTS

Table 1 presents the data on the antimycobacterial activity of *Kappaphycus alvarezii* extracts against $H_{37}R_V$ strain. The results shows that the per cent reduction of RLU in 100 µg/ml was less than that of the control in all the three extracts; the values being -25.46, -15.69 and -42.99 in acetone, chloroform and ethanol, respectively. However, the per cent reduction in RLU was higher in acetone and chloroform extracts at 500 µg/ml concentration. The percent reduction of RLU was +3.90 and +8.91 in both acetone and chloroform extracts. In contrary, in ethanol extract the per cent reduction in RLU was less than that of the control (-31.03). Statistical analysis of the data by Two way ANOVA revealed that the values were significant among various extracts as well as among the two concentrations. The analysis thus depicts that only 500 µg/ml of acetone and chloroform extracts have a significant antimycobacterial activity against $H_{37}R_V$ strain of *Mycobacterium tuberculosis*. Likewise the antimycobacterial activity of *Kappaphycus alvarezii* extracts clinical isolate strain of *Mycobacterium tuberculosis* is presented in Table 2. In our study, the clinical isolate strain responded well to all the three extracts in both the concentrations. The per cent reduction in RLU activity was higher in all the three extracts at 100 µg/ml (+25.90,+59.19, +12.34) and 500 µg/ml (+117.13, +144.89, +64.02). The reduction in RLU activity was directly proportional to the concentrations in all the three extracts. When the data were subjected to Two way ANOVA, the results showed that there was a significant difference only among the concentrations, but the values were on par among various ectracts. The results thus indicates that all the three extracts have antimycobacterial activity against the clinical isolate strain of *Mycobacterium tuberculosis*.

For molecular docking studies, first the 2-D and 3-D structure of kappa-carrageenan was downloaded from ChemSketch database.

Likewise the compound summary of kappa-carrageenan was obtained from PubChem database. Similarly, InhA enzyme sequence was downloaded from SWISSPROT database (Figure 6) in FASTA format and is given below.

FASTA Sequence of InhA Enzyme

>sp|P0A5Y6|INHA_MYCTU Enoyl-[acyl-carrier-protein] reductase [NADH] OS=Mycobacterium tuberculosis GN=inhA PE=1 SV=1
MTGLLDGKRILVSGIITDSSIAFHIARVAQEQ
GAQLVLTGFDRLRLIQRITDRLPAKAPLLEL
DVQNEEHLASLAGRVTEAIGAGNKLDGVV
HSIGFMPQTGMGINPFFDAPYADVSKGIHIS
AYSYASMAKALLPIMNPGGSIVGMDFDPSR
AMPAYNWMTVAKSALESVNRFVAREAGK
YGVRSNLVAAGPIRTLAMSAIVGGALGEEA
GAQIQLLEEGWDQRAPIGWNMKDATPVAK
TVCALLSDWLPATTGDIIYADGGAHTQLL

Domain Analysis

Domain analysis of InhA enzyme was done by using PFAM database (Figure7). The domain analysis revealed that the InhA enzyme belongs to Enoyl-(Acyl carrier protein) reductase domain.

Structure Elucidation of InhA Enzyme

The 3-D crystalline structure of InhA enzyme was downloaded from PDB database (Figure8). The PDB ID is 2H7I.

Ligand Binding Site Prediction

The ligand binding site was predicted using Q-SiteFinder database (Figure 9). The results are as follows:
ILE21,MET103,GLY104,MET147,ASP148,PHE 149,MET155,PRO156,ALA157,TYR158,LYS16 5,VAL189,ALA191,GLY192,PRO193,ILE194,T HR196,MET199,ILE202,LEU207,ALA211,GLN 214,ILE215,LEU218,GLU219.

Grid Generation

For docking kappa-carrageenan with InhA enzyme, first the grid was generated by AutoDock Tools software. The results are given in Figure 10.

Setting Docking Score

The docking scores were set in the AutoDock Tools (Figure 11) and were finally confirmed for docking of the kappa-carrageenan ligand against InhA enzyme (Figure 12).

Docking

Once the scores were set and final confirmation was given the AutoDock software docks the ligand against the InhA enzyme and the docking results are given below in Table 3 and Figure 12. The molecular visualization of the dock was done by Accelrys Discovery Studio visualizer 2.5 software and is presented in Figure 13. A docking score of -11.0 Kcal/mol was obtained. The results show that kappa-carrageenan is effective in docking the InhA enzyme present in both $H_{37}R_V$ (drug sensitive reference strain) and MDR strain (clinical isolate strain) of *Mycobacterium tuberculosis*.

DISCUSSION

Seafood including seaweeds is known to be one of the richest sources of minerals. The most common minerals found in seafood are iodine, magnesium, calcium, phosphorus, iron, potassium, copper and fluoride as stated by Ensminger *et al.* (1995).[27] The authors also added that minerals are very important for the biochemical reaction in the body as a co-factor of enzyme *e.g.*, Ca, P and Mg build and maintain bones and teeth, whereas Na and K help maintain balance of water, acids and bases in fluids outside of cells, and involve in acid-base balance and transfer of nutrients in and out of individual cells, respectively. The infor- mation on carrageenan content of *Kappaphycus alvarezii* remains limited, despite the great importance of this genus in the phycocolloid industry. This fraction has been shown to account for ~14% of the polysaccharide content in other carrageenophytes. It has been reported that total 18 amino acids were found in the dried powder of *Kappaphycus alvarezii*.[28] Among all the amino acids, lysine is the major constituent and followed by asparagines, histidine, isoleucine, phenylalamine, tryptophan. In the case of fatty acids, eight components were identified including two components, namely, palmitic acid and cervonic acid in traces. Alpha linolenic acid (n-3) and linoleic acid are the major components.

Macrominerals were identified by using flame atomic absorption spectrophotometry and it was found that red algae contained various amounts of macrominerals such as sodium (23.4 mg), potasium (12.44 mg), magnesium (23.56 mg), phosphorous (19.5 mg) per 100 mg and rich in calcium (3.565 gm/100 gm). The studies showed that red seaweeds could be used as a food supplement to meet the recommended daily intake of some essential minerals. From the overall study, the authors concluded that *Kappaphycus alvarezii* can serve as functional food with vital nutritional and biological values. Several authors have worked on various aspects of *Kappaphycus alvarezii*. Screening of antimicrobial activities in red, green and brown macroalgae from Gran Canaria (Canary Islands, Spain) has been done by deVal *et al.* (2001).[29] Chemical structure and antiviral activity of carrageenans from *Meristiella gelidium* against *Herpes simplex* and *Dengue virus* was assessed by deTischer *et al*. (2006).[30] Phycoremediation of heavy metals cadmium, cobalt and chromium by the three-colour forms of *Kappaphycus alvarezii* has been carried out by Kumar *et al*. (2007).[31] Antibacterial activity of the extracts of marine red and brown algae on *Pseudomonas fluorescence*, *Staphylococcus aureus*, *Vibrio cholera*, *Proteus mirabilis* has been studied by.[32] The potential application of *Kappaphycus alvarezii* in agicultural and pharmaceutical industry has been reviewed.[28] Similarly, antibacterial activity of *Sargassum Ilicifolium* and *Kappaphycus alvarezii* was carried out by Rebecca *et al*.[33] On the other hand, work on antimycobacterial activity of *Kappaphycus alvarezii* has not been carried out till date. In the present study, two strains of *Mycobacterium tuberculosis* were treated with 100 µg/ml and 500 µg/ml of acetone, chloroform and ethanol extracts of *Kappaphycus alvarezii* and the inhibiting property of the drug was assessed using LRP assay. The results showed that acetone and chloroform extracts of *Kappaphycus alvarezii* at 500 µg/ml concentration showed good inhibition in the activity of the $H_{37}R_V$ strain. In contrary, when the drug was tested against clinical isolate strain, an inhibition in the activity was recorded in both the concentrations of the drug. The results suggests that the drug even at lower concentration, inhibits the activity of clinical isolate strain, but the activity of multi-drug resistant strain was inhibited only at higher concentration. So *Kappaphycus alvarezii* proves to be an antimycobacterial agent against *Mycobacterium tuberculosis*. Further, to find out the mode of action of *Kappaphycus alvarezii*, used molecular docking studies by using kappa-carrageenan, the active component of *Kappaphycus alvarezii* as a ligand against InhA enzyme present in both strains of *Mycobacterium tuberculosis*. For this, the three dimensional structure of the receptors was downloaded from PDB Database. The active sites of receptors were identified using Q-SiteFinder. The 3D structures of InhA enzyme were docked with kappa-carrageenan inhibitor using AutoDock Software. The docking results were analyzed using Accelrys Discovery Studio Visualization Tool. From the above docking studies, it was found out that kappa-carrageenan formed one hydrogen bond with the docking score of -11.5 with InhA enzyme. The results revealed that *Kappaphycus alvarezii* is highly effective in controlling *Mycobacterium tuberculosis*.

CONCLUSION

The present reveals that *Kappaphycus alvarezii* has inhibitory activity against *Mycobacterium tuberculosis* and to elucidate the medicinal properties of *Kappaphycus alvarezii*, especially the active component *viz*., kappa-carrageenan, there is a need for further investigation that will pave a way for finding this mrine bioresource as a medicine to control tuberculosis.

ACKNOWLEDGEMENT

The authors like to thank Dr. Mrs. Vanaja Kumari, Principal Scientist F, Bacteriology Department and Dr. Prabhu Srinivasan, Consultant Microbiologist, Tuberculosis Research Centre, (ICMR), Chetpet, Chennai, for allowing to work in their esteemed organization and providing me with mycobacterium strains and we are extremely thankfull to M/S Helix

Infosystems, Chennai, for their technical guidance and allowing to carryout bioinformatics techniques in their Institute and providing software.

Table 1: Antimycobacterial activity of *Kappaphycus alvarezii* extracts against $H_{37}R_V$ strain of *Mycobacterium tuberculosis*

S. No.	Compound	% of reduction in RLU (100 µg/ml)	% of reduction in RLU (500 µg/ml)
1	Control (Rifampicin)	81.90 [ab]	81.90 [ab]
2	Acetone extract	61.05 [ab] (-25.46)	85.09 [ab] (+ 3.90)
3	Chloroform extract	69.05 [ab] (-15.69)	89.20 [ab] (+8.91)
4	Ethanol extract	46.69 [ab] (-42.99)	56.49 [ab] (-31.03)

Values in parantheses denotes per cent change over control.
- Denotes per cent decrease than that of the control.
+ Denotes per cent increase than that of the control.
Values in superscript denotes significance at 5% level (Two way ANOVA).
[a] Denotes that values are significant among various extracts.
[b] Denotes that values are significant among various concentrations.

Table 2: Antimycobacterial activity of *Kappaphycus alvarezii* extracts against clinical isolate strain of *Mycobacterium tuberculosis*

S. No.	Compound	% of reduction in RLU 100µg/ml	% of reduction in RLU 500µg/ml
1	Control (Rifampicin)	34.44 [b]	34.44 [b]
2	Acetone extract	43.36 [b] (+25.90)	74.78 [b] (+117.13)
3	Chloroform extract	54.48 [b] (+59.19)	84.34 [b] (+144.89)
4	Ethanol extract	38.69 [b] (+12.34)	69.26 [b] (+64.02)

Values in parantheses denotes per cent change over control.
+ Denotes per cent increase than that of the control.
Values in superscript denotes significance at 5% level (Two way ANOVA).
b Denotes that values are significant among various concentrations.

Table 3: Docking results of kappa-carrageenan against InhA enzyme

InhA Enzyme		Kappa-Carrageenan	Distance (Å)	Docking Score (Kcal/Mol)
Residue	Atom			
ILE21	HN	O	2.08	Final Docked Energy -7.03 Final Intermolecular Energy -10.76 Final Internal Energy of Ligand -0.90 Torsional Free Energy +3.74

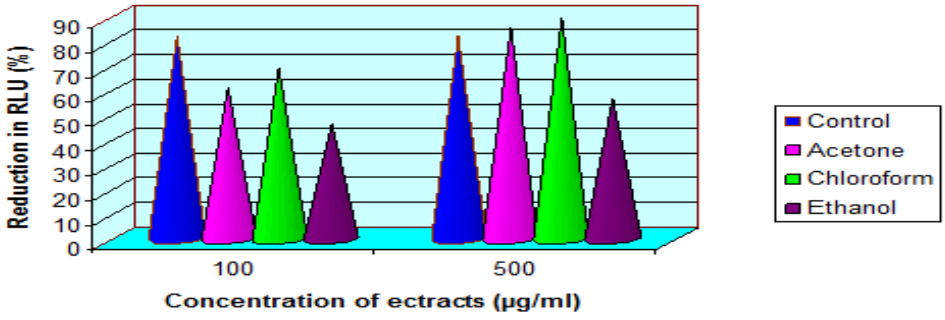

Figure 1: Antimycobacterial activity of *Kappaphycus alvarezii* extracts against $H_{37}R_V$ strain of *Mycobacterium tuberculosis*

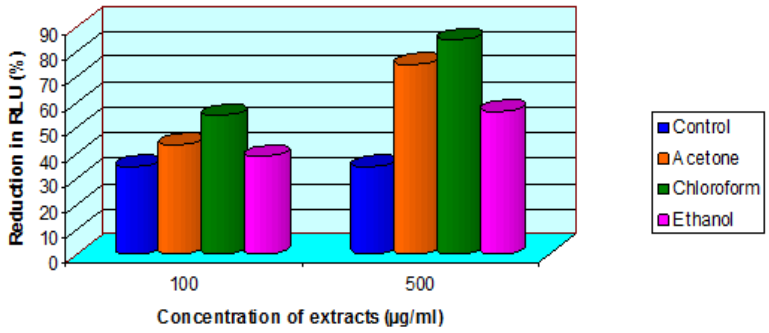

Figure 2: Antimycobacterial activity of *Kappaphycus alvarezii* extracts against clinical isolate strain of *Mycobacterium tuberculosis*

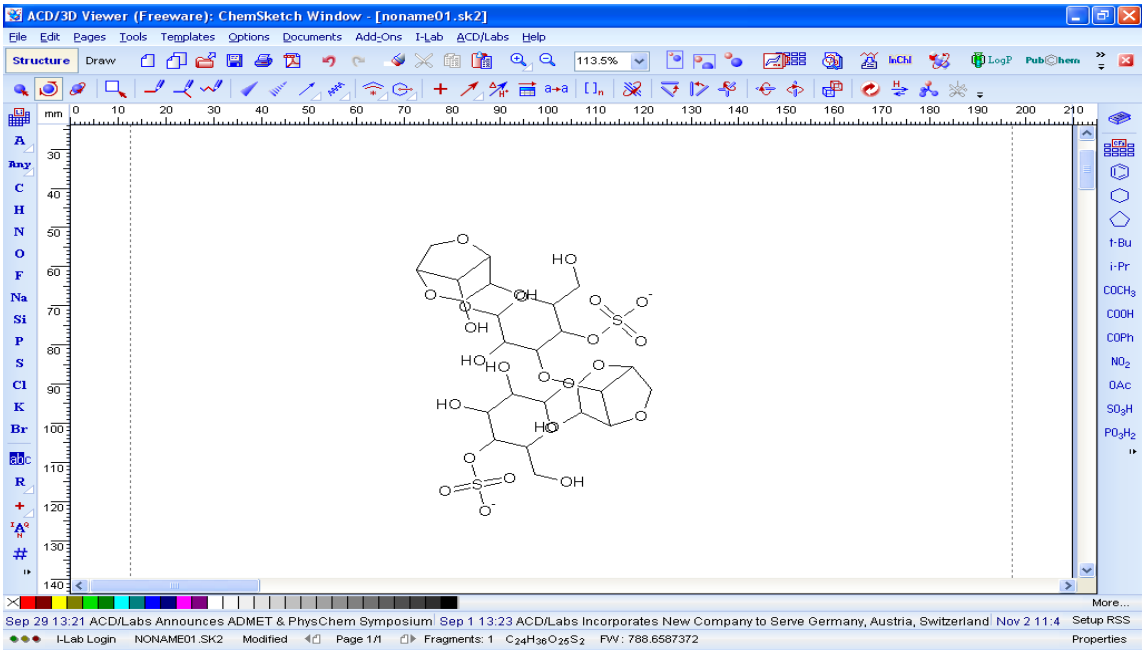

Figure 3: 2D Structure of Kappa-Carrageenan

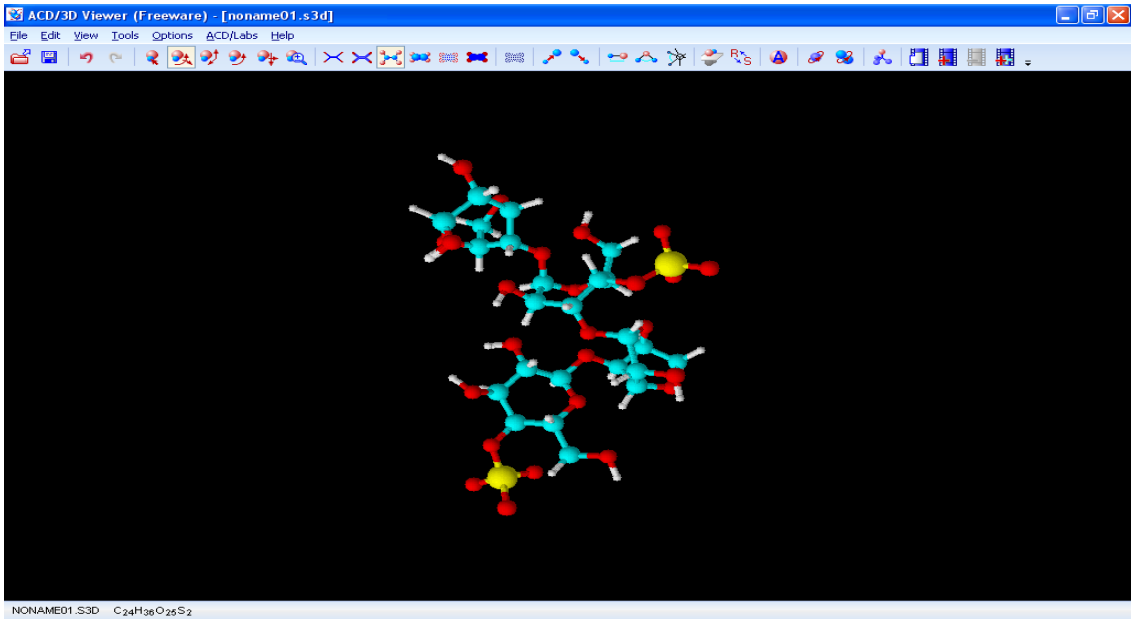

Figure 4: 3D Structure of Kappa-Carragennan

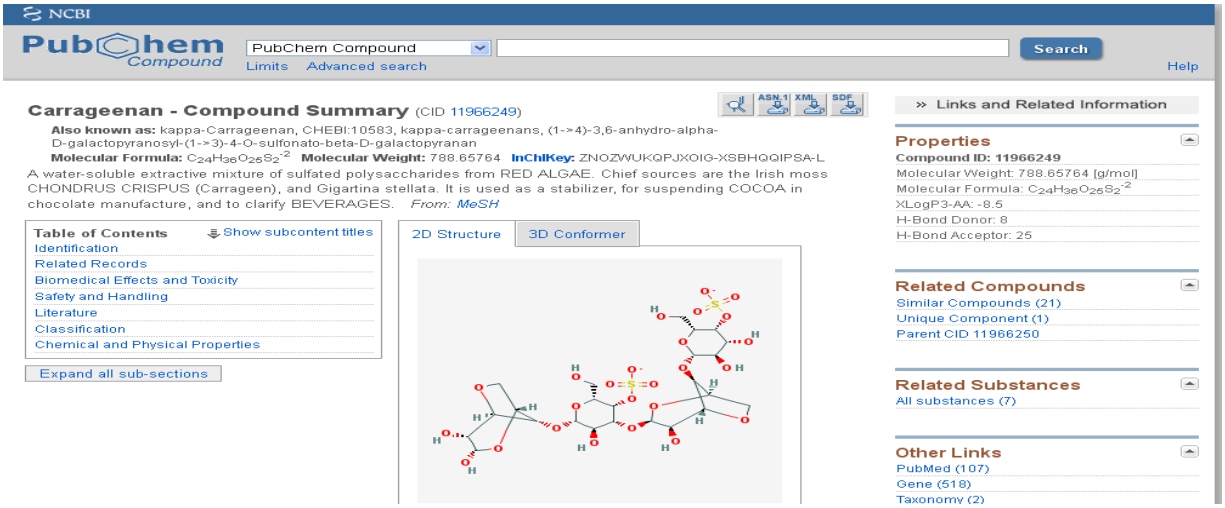

Figure 5: PUBCHEM Homepage

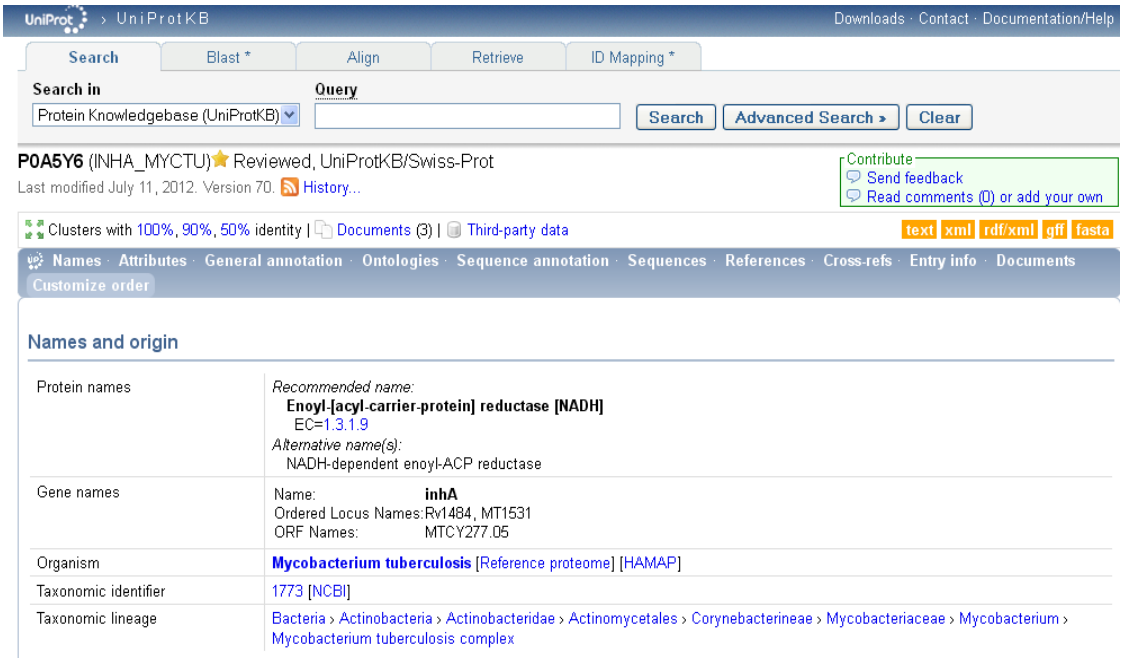

Figure 6: SWISSPROT Result Page

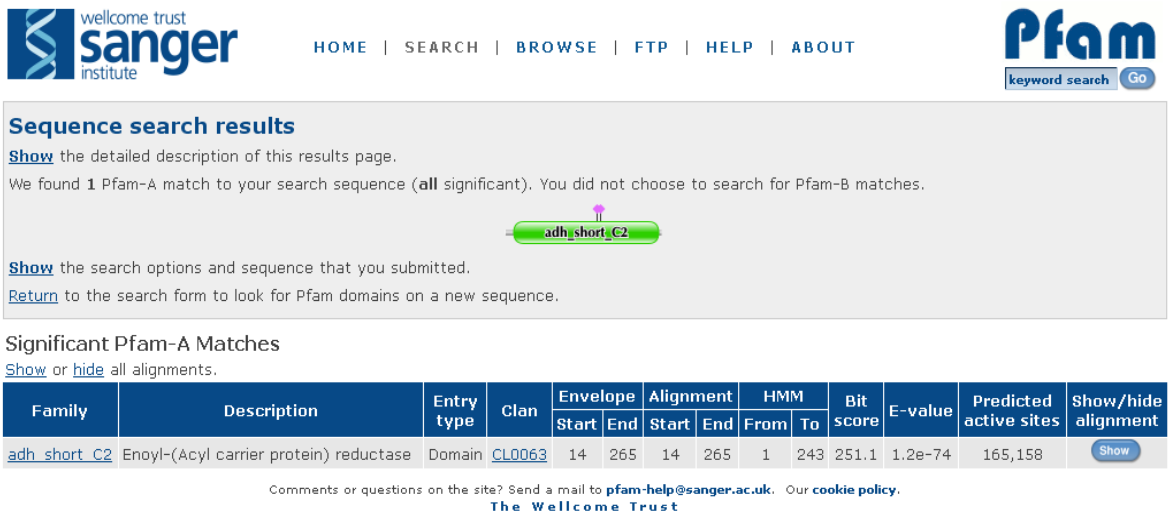

Figure 7: PFAM Domain Analysis

Figure 8: PDB Result Page

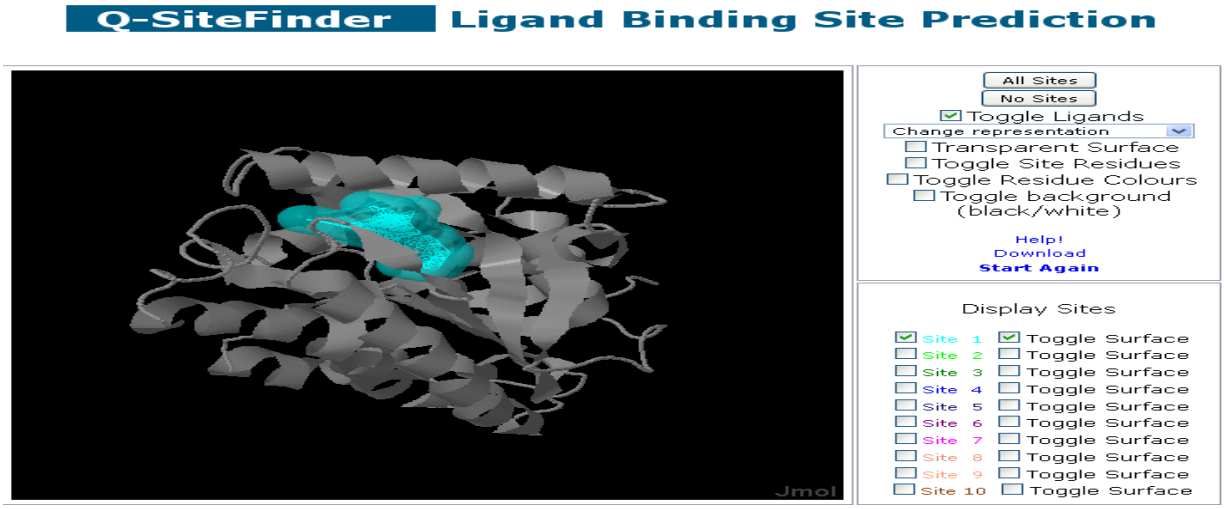

Figure 9: Q-SiteFinder Result Page

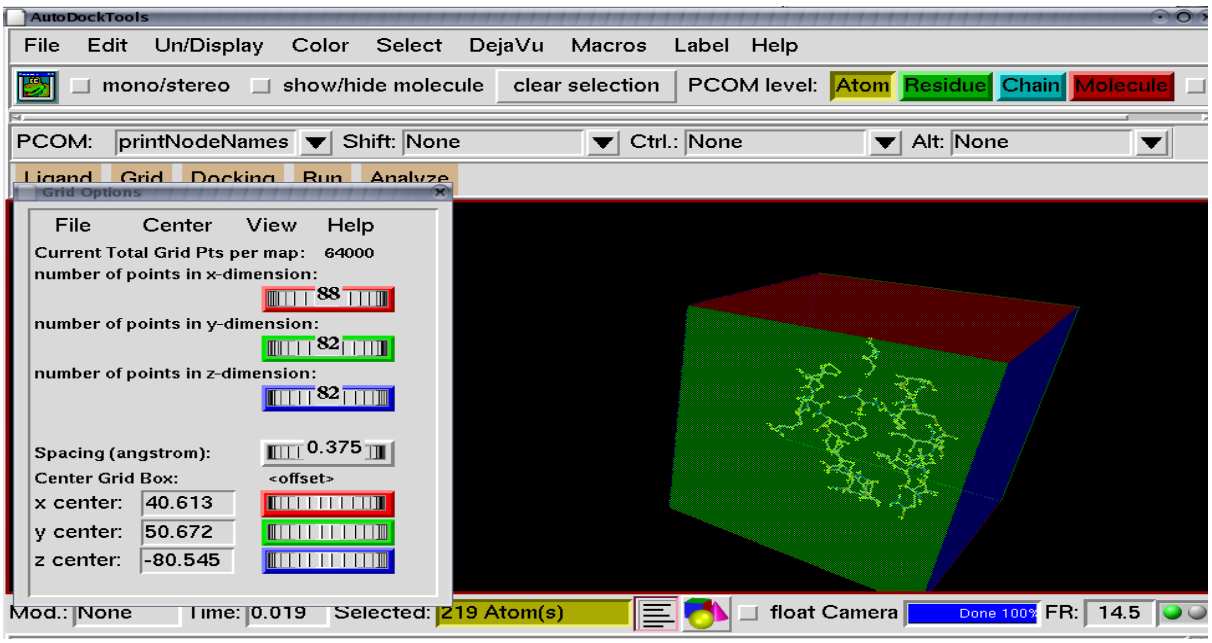

Figure 10: Kappa-Carrageenan Interation with InhA Enzyme

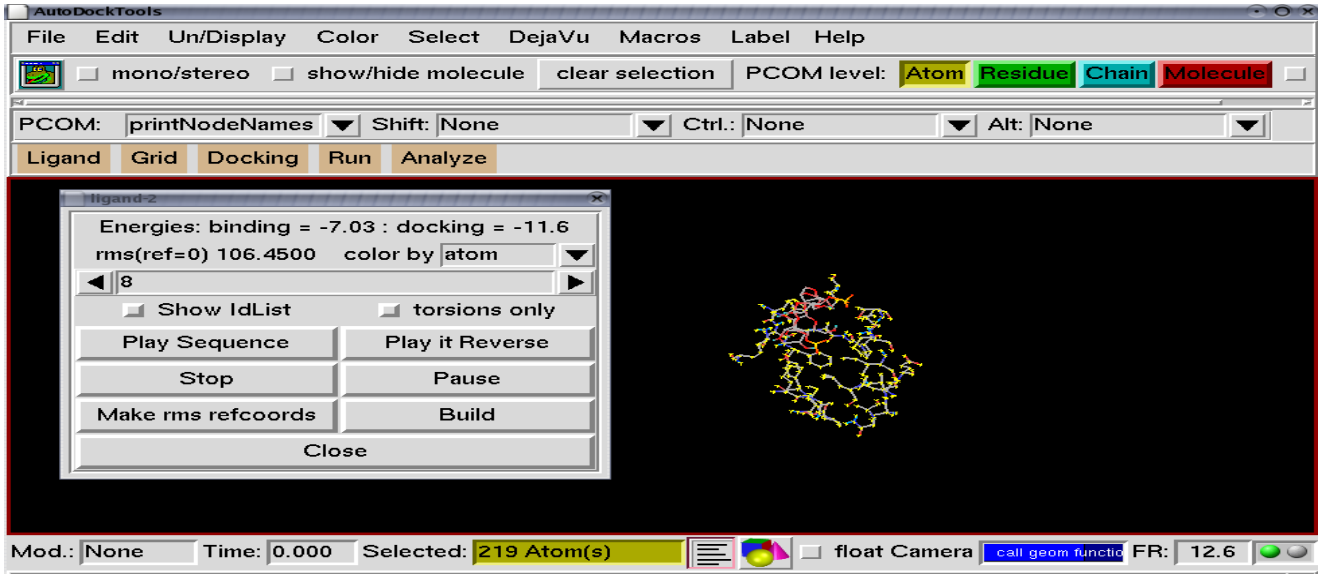

Figure 11: Setting Docking Score

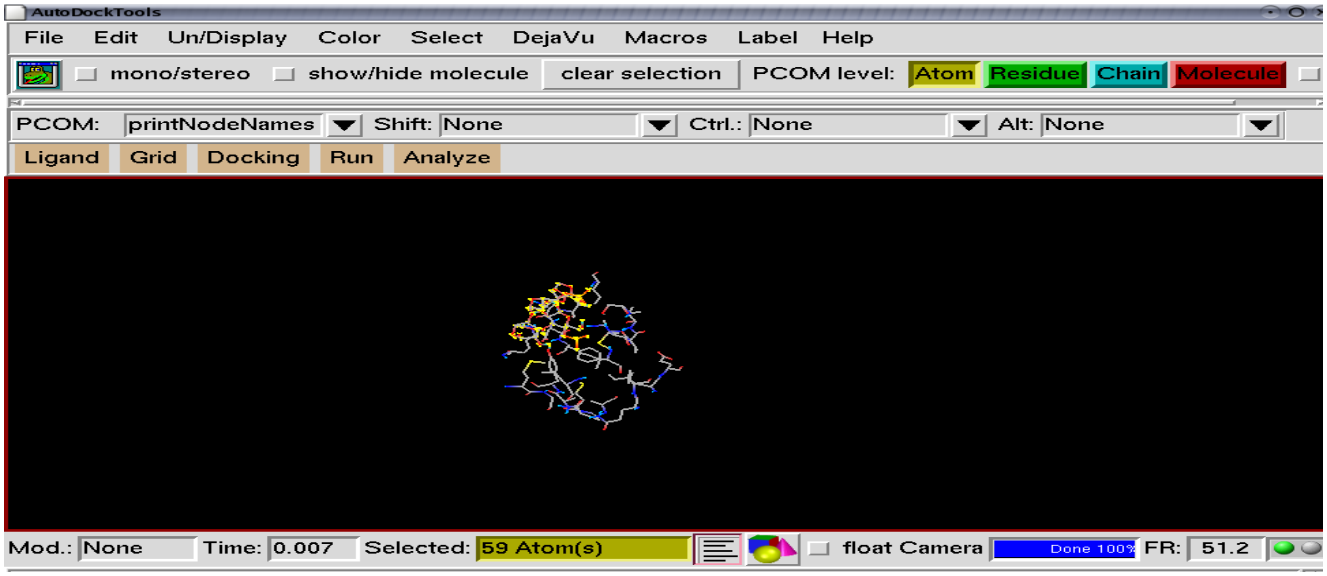

Figure 12: Final Conformation of Docking

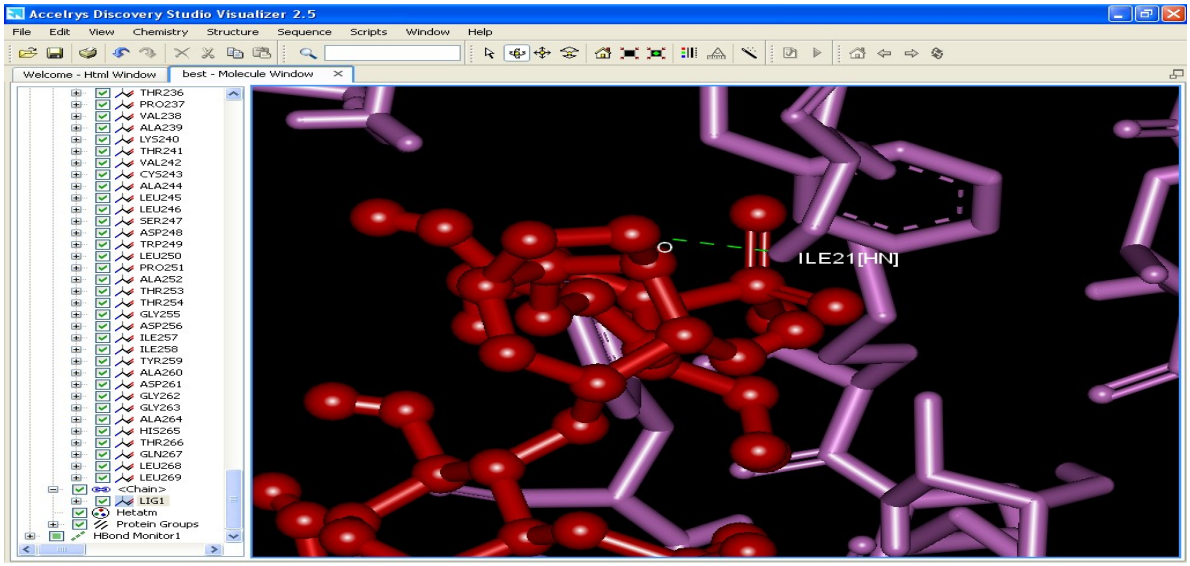

Figure 13: Docking of kappa-carrageenan ligand against InhA enzyme

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Cite This Article: A, Mayakrishnan; K, Sivakumari1; K, Ashok; Prabhu, Srinivasan and Vanaja, Kumari (2015), "Antimycobacterial activity of *Kappaphycus alvarezii* against *Mycobacterium tuberculosis* and *In Silico* molecular docking of kappa-carrageenan against InhA enzyme", ***International Journal of Drug Research and Technology***, Vol. 5 (1), 35-46.

INTERNATIONAL JOURNAL OF DRUG RESEARCH AND TECHNOLOGY