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# Biochemical evaluation and molecular docking studies on encapsulated astaxanthin for the growth inhibition of *Mycobacterium tuberculosis*

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**ABSTRACT**

Astaxanthin, a tetraterpenoid compound was formulated as liposomes and screened for anti-tubercular activity against different strains of *Mycobacterium tuberculosis*: MTB, MDR-TB, and H37Rv isolated from the patient sputum samples. Minimum inhibitory concentration (MIC) was determined by proportion-based method. The molecular docking analysis of astaxanthin with various mycobacterial drug target proteins were performed using *in silico* tools. Encapsulated astaxanthin showed better inhibition of MTB with MIC value of 500 µg/ml against all the selected strains. The docking analysis provided the interacting amino acid residues of selected mycobacterial enzymes and mode of ligand–protein interaction. Based on the present study, it was concluded that encapsulated astaxanthin could be a better cost-effective natural compound for the treatment of tuberculosis among the patients which will combat side effects in comparison with the first line AntiTB drugs like rifampicin and isoniazid.

## 1. INTRODUCTION

Mycobacterial infections and multidrug-resistant (MDR) strains of *Mycobacterium* generate high mortality and socio-economic burdens throughout the world. *Mycobacterium tuberculosis* (MTB) is one of the first and foremost human pathogens which lead to cause tuberculosis (TB) [1]. The organs such as lungs, central nervous system, lymphatic system, and circulatory system get damaged due to this disease [2]. Host's inherited susceptibility, environmental risk factors, and genetic variations were responsible for the progression of MTB [3]. Every year, 8 million new TB cases were stated and over 2 million deaths occur [4] and as per WHO report, one-third of the worldwide population has been at risk of infection with MTB [5]. The anti-tubercular drugs have become less effective due to emergence of extensively drug resistant (XDR) strains of MTB [6].

MDR-TB is mainly elevated through strains that are resistant to the anti-TB drugs such as isoniazid and rifampicin. MDR-TB raises either due to the infection with MTB which is already drug-resistant or may perhaps develop in the course of a patient's treatment. Widely, drug-resistant TB (XDR-TB) is a form of TB which is arised by organisms resistant to isoniazid and rifampicin as well as any fluoroquinolone and may also due to second-line anti-TB injectable drugs (amikacin, kanamycin). These forms of TB can take 2 years or more for the treatment when given with drugs that are less potent, more toxic and much more expensive. The drug resistant strains do not respond to the first 6 months of treatment with first-line anti-TB drugs.

The first- and second-line antimycobacterial drugs like isoniazid, pyrazinamide are the group of nitrogen-based heterocyclic compounds and most of them are the derivative of pyridine and

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pyrazines [7]. Many chalcone derivatives, carboxamide, imidazole, and benzimidazole derivatives were also reported to inhibit drug-resistant strains [8]. The physico-chemical, metabolic, and pharmacokinetic properties of all benzimidazoles with different heterocyclic substituents led to essential modification of strains which could be essentially due to the resistance developed by the mycobacterial strains [9]. The mechanism of action of all the available drugs on the growth of MTB is through their inhibitory effect on bacterial cell wall synthesis, folic acid metabolism and replication machinery of the bacteria. However, due to poor potency, instability, low solubility, and decreased bioavailability amongst the drugs; there is always a thirst for the discovery of novel drugs against MDR MTB.

Astaxanthin, a red colour pigment belongs to the group of xanthophyll, which is the oxygenated derivative of carotenoids. This pigment predominantly occurs in crustacean, salmonids and other farmed fish feeds. The major role of astaxanthin is to provide the desirable reddish-orange color to many organisms as they do not have access to natural sources of carotenoids [10]. Astaxanthin has been found in numerous micro-organisms Such as *Haematococcus pluvialis*, *Chlorella zofingiensis*, and *Chlorococcum* sp., the red yeast *Phaffiarhodozyma*, and the marine bacterium *Agrobacterium aurantiacum*. The potential health-promoting effects of astaxanthin in the prevention and treatment of various diseases, such as cancers, diabetes, cardiovascular diseases, etc. have been proved by several studies [11]. Basically, the chemical structure of Astaxanthin contains keto-Carotenoid with two hydroxyl groups and carbonyl functional groups in each ionone ring. The chemical structure of astaxanthin with conjugated double bond system deliberates to get esterified, increases its polarity and makes it to produce good antioxidant (Fig 1). By converting astaxanthin to get esterified with protein or fatty acid in the biological system, the instability and oxidation of astaxanthin in free form can be overcome. The stability was also enhanced by microencapsulation of astaxanthin as microspheres emulsions, gels, beads, and liposomes [12].

Ionone ring characteristic to carotenoids was also reported to have potent antibacterial and antifungal activity. Furthermore, encapsulation as liposomes increase the lipophilic nature, stability, and bioavailability. Liposomal encapsulation of astaxanthin increases their therapeutic potential against oxidant-induced tissue injuries, because liposomes facilitate intracellular delivery and prolong the retention time of entrapped agents inside the cell [13]. Furthermore, the research towards treatment of MTB infections by using natural products exhibiting low side effects and identification and characterization of new drug targets becomes inevitable. Hence, the aim of the present study is to investigate the anti-mycobacterial activity of both non-encapsulated astaxanthin and encapsulated astaxanthin as liposomes against MDRTB, MTB, $H_{37}$RV strains and to dock the astaxanthin and positive drug Rifampycin and with essential proteins: [Enoyl-Acyl Carrier Protein (ACP) Reductase], 3-Oxoacyl-[Acyl-Carrier Protein] Reductase, Pantothenate kinase, and Isocitrate dehydrogenase.

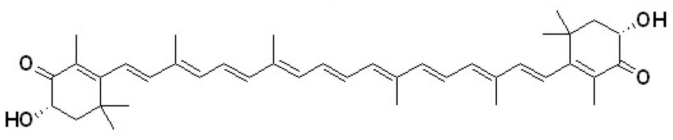



**Figure 1:** Structure of astaxanthin.

## 2. MATERIALS AND METHODS

### 2.1. Sample for Testing Anti-Tuberculosis Activity

The *in vitro* evaluation of anti-TB test was carried out using encapsulated and non-encapsulated Astaxanthin. The standard Astaxanthin was procured from Rudra Bioventures Pvt Ltd., Bangalore and encapsulated as liposomes using L. Phosphatidyl Choline and Cholesterol and physicochemical characterization was done and reported [14].

### 2.2. Source of Mycobacterial Strains and Preparation of Inoculums

The sputum samples were received from District Tuberculosis Center, MGM hospital, Warangal. The sample was inoculated with the Lowenstein-Jensen (LJ) solid medium and kept for incubation at Department of Microbiology, Sri Shivani College of Pharmacy for 8 weeks. The cultures were identified as MTB based on morphological and biochemical methods of which MTB and MDR-TB were studied. Further, the clinical isolates were preserved at − 20°C in liquid broth containing 10% glycerol; retrieval of the culture was carried by sub culturing on sterile L-J medium and incubated for a period of 8 weeks. At 4th week, culture was used to prepare the Mycobacterial suspension in middle brook media, according to McFarland turbidity method. The bacterial suspension was homogenized by vortex shakeup and the turbidity was adjusted in agreement with tube according to McFarland no.1 scale (3.2 × 106 cfu/ ml). The inoculum was prepared by diluting the bacterial mixture solution in the ratio of 1:20 in Middle Brook 7H9 broth medium. Then, the screening of drug activity was carried out by inoculating this diluted suspension (100 µl) [15]. For testing of antimycobacterial activity of the test compounds, MTB $H_{37}$RV was taken as a control strain.

### 2.3. L-J Media Preparation

For the isolation and differentiation of MTB, L-J medium is used with fresh egg and glycerol. L-J medium containing glycerol promote the growth of MTB.

### 2.4. Middle Brook Media (Liquid media)

The liquid growth medium used for culture of MTB is Middle brook 7H9 Broth which was purchased from Himedia Labs Pvt., Ltd., Mumbai. The composition of the media includes Ammonium sulphate 0.05%, Disodium phosphate 0.25%, Monopotassium phosphate 0.1%, Sodium citrate 0.01%, Magnesium sulphate 0.005%, Calcium chloride 0.00005%, Zinc sulphate 0.0001%, Copper sulphate 0.0001%, Ferric ammonium citrate 0.004%, L-Glutamic acid 0.05%, Pyridoxine 0.0001%, and Biotin 0.00005% [16].

### 2.5. Glycerol and OADC

Glycerol (2 ml) added to the media, dissolves the ingredients by heating and autoclave at 121ºC for 15 minutes to sterilize this

solution keep indefinitely cool it. Bovine Albumin Fraction v (2.50 gm), Dextrose (1.00), Catalyse (0.002 gm), Oleic acid (0.025 gm), Sodium chloride (0.425 gm), distilled water (50 ml). The OADC were added to the cool medium under aseptic condition. Within 5–7 days after inoculation and once in a week up to 21 days, the cultures were read spectrophotometrically at 450 nm [16].

### 2.6. Preparation of Stock Solution for Screening Antimycobacterial Activity

The sample under study, astaxanthin and encapsulated astaxanthin were dissolved in methanol and stock solution of concentration 1 mg/ml was prepared. The test compounds were diluted to various concentrations ranging from 4 µg/ml –to 1,000 µg/ml to determine MIC against all the tested strains [16].

### 2.7. Culture Inoculation of Test Samples and Mycobacterium

Specific concentrations of test compounds were added individually to the freshly prepared sterile media and mixed well. A loop of diluted Mycobacterial subculture and control were inoculated into sterile medium separately in respective tubes under aseptic conditions and mixed properly using Vortex. The culture tubes were incubated at 37 °C over the period of growth. The result analysis was performed visually after 3rd, 5th, 7th, 9th, 15th, and 21st days of stationary incubation at 37°C. The lowest concentration of the compound at which no visible bacterial growth was observed was considered as MIC's [16].

### 2.8. Confirmation of Antimycobacterial Activity

Anti-mycobacterial activity of encapsulated and non-encapsulated astaxanthin was assessed by the presence of turbidity on 7th, 9th, 15$^{th}$, and 21st day and was confirmed by smear method and biochemical test (catalase test) after 21 days of incubation.

### 2.9. *In silico* Methods

#### *2.9.1. Molecular docking*

From PubChem (https://pubchem.ncbi.nlm.nih.gov/), the 3D structure of astaxanthin and positive anti-TB drugs were retrieved (Table 1). Likewise, the 3D structures of different Mycobacterial Proteins were collected from Protein data bank (www.rcsb.org) (Table 2). The drug targets were chosen based on literature review. Mycobacterial enoyl-ACP-reductase and 3-Oxoacyl-[ACP] Reductase were key enzymes that regulate mycolic acids biosynthesis, has been established as promising target for the discovery of novel antimycobacterial drugs. Pantothenate Kinase regulates the rate limiting step of CoA biosynthesis. Isocitrate dehydrogenase (ICD), a key regulatory enzyme in the citric acid cycle, was among the marker enzyme released in late log phase of growth cycle of *Mycobacterium*. Hence, all these enzymes which were required for the bacteria for survival was chosen. The inhibitory role of astaxanthin on these enzymes were evaluated by molecular docking. Isoniazid and rifampicin which act as positive drugs for TB were also used for docking process and the binding efficiency was compared with the binding capacity of Astaxanthin with anti-TB drugs.

**Table 1:** Compound name with PubChem CID for molecular docking.

| S.NO | Compound name | PubChem CID | Molecular formula | Molecular weight |
|---|---|---|---|---|
| 1. | Astaxanthin | 5281224 | $C_{40}H_{52}O_4$ | 596.852 g/mol |
| 2. | Isoniazid | 3767 | $C_6H_7N_3O$ | 137.139 g/mol |
| 3. | Rifampicin | 5381226 | $C_{43}H_{58}N_4O_{12}$ | 822.953 g/mol |

**Table 2:** Mycobacterial proteins with PDB ID.

| S.NO | Receptors | PDB ID |
|---|---|---|
| 1. | Protein (Enoyl-Acyl Carrier Protein (ACP) Reductase) | 1BVR |
| 2. | 3-Oxoacyl-[Acyl-Carrier Protein] Reductase | 1UZN |
| 3. | Pantothenate kinase | 3AF3 |
| 4. | Isocitrate dehydrogenase | 5KVU |

**Table 3:** Docking parameters.

| S.NO | Protein clean | |
|---|---|---|
| 1. | *tstep* | 0.2 |
| 2. | *qstep* | 5.0 |
| 3. | *dstep* | 5.0 |
| 4. | *rmstol* | 2.0 |
| 5. | *ga_pop_size* | 150 |
| 6. | *ga_num_evals* | 250,000 |
| 7. | *ga_num_generations* | 540,000 |
| 8. | *ga_run* | 10 |

Docking Server (https://www.dockingserver.com/web) was used for docking calculations [17]. Gasteiger partial charges were added to the ligand atoms. Non-polar hydrogen atoms were combined, and rotatable bonds were well-defined. For Astaxanthin-protein model the docking calculations were carried out. Essential hydrogen atoms, Kollman united atom type charges, and solvation parameters were added with the support of AutoDock tools [18]. An AutoDock parameter set and distance-dependent dielectric function was used in the calculation of the van der Waals and the electrostatic terms. Docking models were carried out using the Lamarckian genetic algorithm and the Solis & Wets local search method [19]. Initial position, orientation, and torsions of the ligand molecules were set randomly. During docking, all rotatable torsions were released. Each docking experiment was derived from 10 different runs (*ga_run*) that were set to terminate after a maximum of 250,000 energy evaluations (*ga_num_evals*). The population size was set to 150 (*ga_pop_size*). During the search, a translational step of 0.2 Å (*tstep*), and quaternion and torsion steps of 5 (*qstep*) were applied (Table 3). The overall binding potential was assessed by the interaction profile and binding energy of Astaxanthin when compared with anti-TB drugs.

## 3. RESULTS

The size of liposomal microencapsulated astaxanthin was characterized using scanning electron microscopy as shown in Figure 2. The size of the particle ranged from 7.211 to 15.56 µm which shows that size of microencapsulated astaxanthin increases when liposomal agents (L-phosphatidylcholine and cholesterol)

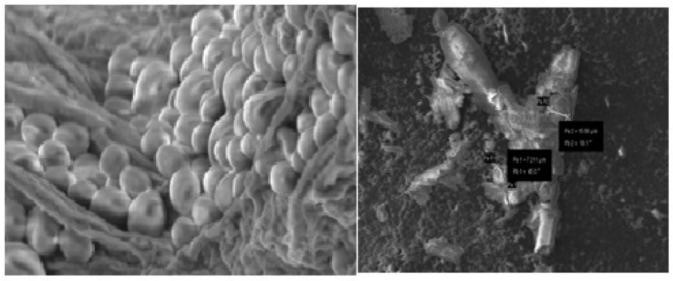


**Figure 2:** SEM image of liposomal encapsulated astaxanthin.

were used. In our previous study, the structure of microencapsulated astaxanthin using different encapsulating agents was predicted, characterized, and reported [14].

The antimycobacterial activity of test drugs such as non-encapsulated and liposomal encapsulated astaxanthin was screened in liquid media and MICs were determined against MTB, MDRTB, and $H_{37}$RV strains. At concentration 500 µg/ml, growth was observed for all the three strains (MDRTB, MTB, and $H_{37}$RV) in the tubes treated with non-encapsulated astaxanthin. Confirmatory tests such as smear microscopy shows that non-encapsulated astaxanthin produce positive (+ve) for the growth of $H_{37}$RV, MTB, MDR-TB strains at concentration 500 µg/ml which was evident by growth of culture, and Biochemical test such as catalase test also showed positive at 500 µg/ml concentration which was indicated by bubbles in tubes inoculated with samples. Thus, the non-encapsulated drug was inactive for anti-mycobacterial activity. On a contrary, no turbidity was found in the tubes treated with liposomal encapsulated astaxanthin at 500 µg/ml concentration with all the tested strains, viz., MDRTB, MTB, and $H_{37}$RV. Smear test and biochemical test shows that liposomal encapsulated astaxanthin give negative result which is evident with the inhibition in growth of colonies after 21 days. Thus, the drug after encapsulating as liposomes was found to be active towards various *Mycobacterium* strains (Table 4 and Fig. 3). The growth inhibitory potential of astaxanthin over mycobacterial strains was observed and the result has been interpreted. Since, astaxanthin have many roles in pharmacological studies, the present research work was carried out to discover the *in vitro* anti-mycobacterial activity of encapsulated and non-encapsulated astaxanthin based on medicinal uses.

The results of molecular docking analysis were given in terms of binding energy and the pose with lowest binding free energy (Table 5). The binding efficiency of small molecules to the active-site of various protein targets were assessed in terms of least free energy of binding. The binding mode and active site interacting of amino acids between 3D structure of astaxanthin, isoniazid, and rifampicin with mycobacterial proteins 1BVR, 1UZN, 3AF3, and 5KVU was shown in Figures 4a to 4d, 5a to 5d, and 6a to 6d, respectively.

Astaxanthin was found to establish polar, hydrophobic, and other weak interaction with all the target proteins. Astaxanthin showed better binding efficiency as analyzed with least free energy of binding and many hydrophobic bonds established with all the target proteins. The binding pocket and interacting amino acid residues varied with all the individual proteins.

The interaction between isoniazid and different *Mycobacterium* proteins gained lower binding energy of −4.68 kcal/mol (PDB: 1BVR), −4.34 kcal//mol (PDB: 1UZN), −4.53 kcal/mol (PDB: 3AF3) and −4.50 kcal/mol (PDB: 5KVU) with inhibition constant

**Table 4:** Confirmation of anti-mycobacterial activity of encapsulated and non-encapsulated Astaxanthin based on smear and biochemical tests.

| Compound name | MIC Conc (µg/ml) | Strain | | | | | | Final report |
|---|---|---|---|---|---|---|---|---|
| | | MTB | | MDRTB | | H37RV | | |
| | | Smear test | B.C test | Smear test | B.C test | Smear test | B.C test | |
| Non-encapsulated astaxanthin (Standard) | 500 | + ve | + ve | + ve | + ve | + ve | + ve | Inactive towards all the strains |
| Encapsulated Astaxanthin | 500 | ~ ve | ~ ve | ~ ve | ~ ve | + ve | + ve | Active toward MTB and MDRTB but not towards H37RV |

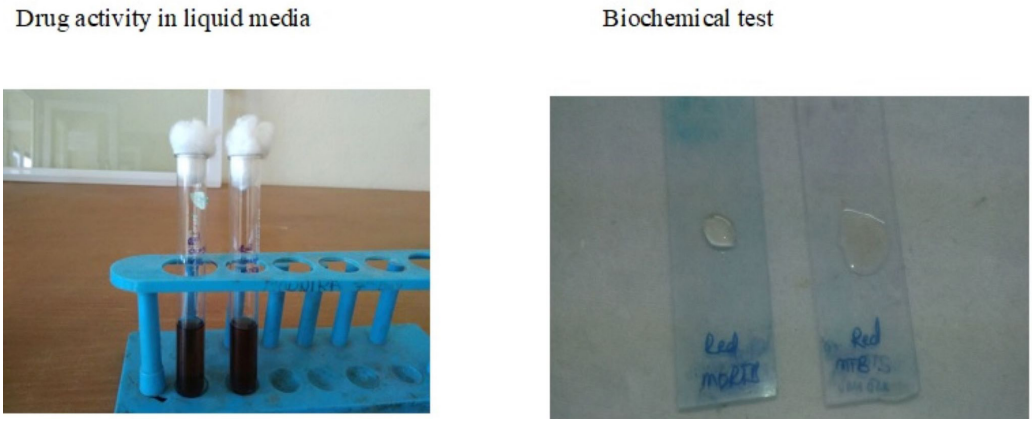
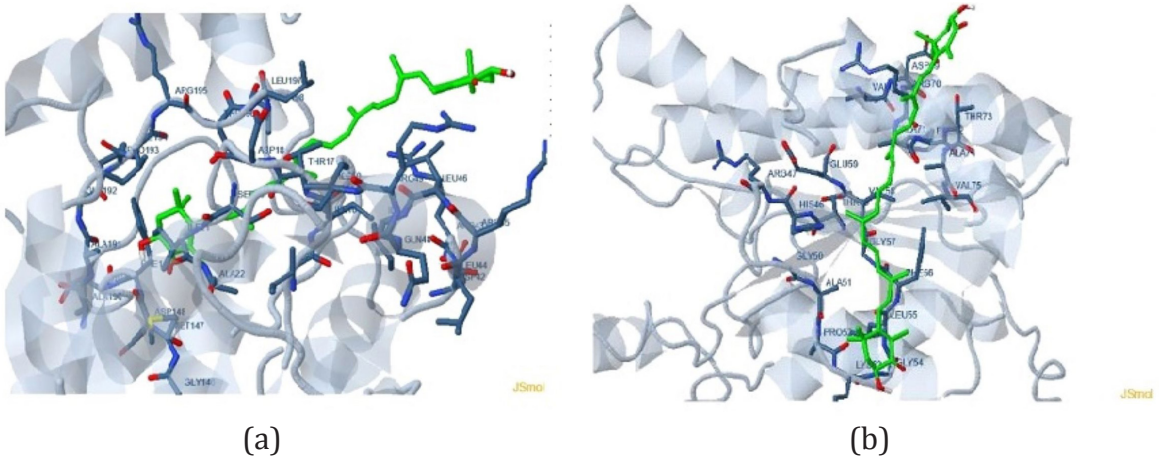



**Figure 3:** Biochemical test for *Mycobacterium. tuberculosis*.

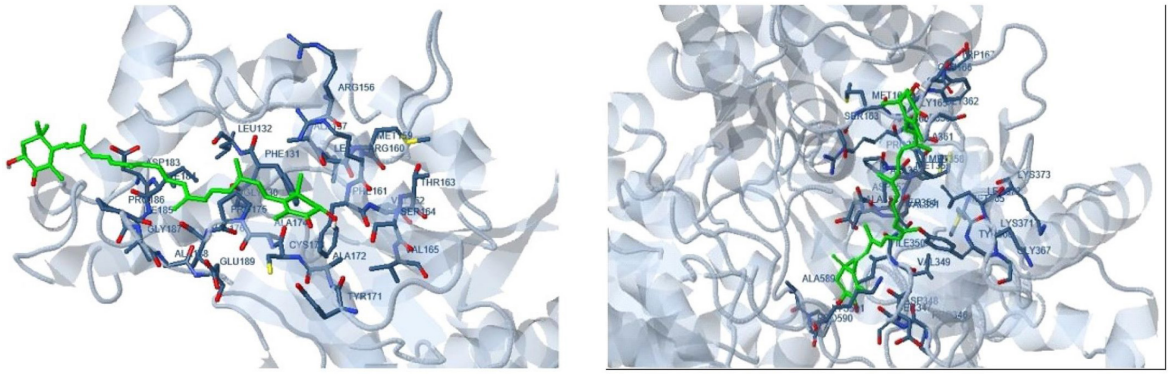
**Table 5:** Molecular docking results with binding energy.

| Docking | Est. free energy of binding | Est. inhibition constant, Ki | vdW + Hbond + desolv energy | Electrostatic energy | Total intermolecular energy | Frequency | Interaction surface |
|---|---|---|---|---|---|---|---|
| Astaxanthin + 1BVR | − 5.56 kcal/mol | 83.53 µm | − 9.02 kcal/mol | − 0.13 kcal/mol | − 9.15 kcal/mol | 10% | 1,148.959 |
| Astaxanthin + 1UZN | − 5.43 kcal/mol | 105.00 µm | − 8.28 kcal/mol | − 0.03 kcal/mol | − 8.13 kcal/mol | 10% | 892.728 |
| Astaxanthin + 3AF3 | − 5.16 kcal/mol | 166.00 µm | − 7.95 kcal/mol | + 0.01 kcal/mol | − 7.94 kcal/mol | 10% | 929.643 |
| Astaxanthin + 5KVU | − 6.26 kcal/mol | 25.76 µm | − 9.23 kcal/mol | + 0.04 kcal/mol | − 9.19 kcal/mol | 10% | 1,650.607 |
| Isoniazid + 1BVR | − 4.68 kcal/mol | 370.36 µm | − 5.40 kcal/mol | − 0.10 kcal/mol | − 5.50 kcal/mol | 10% | 409.792 |
| Isoniazid + 1UZN | − 4.34 kcal/mol | 659.54 µm | − 4.86 kcal/mol | − 0.10 kcal/mol | − 4.96 kcal/mol | 20% | 420.82 |
| Isoniazid + 3AF3 | − 4.53 kcal/mol | 477.93 µm | − 5.09 kcal/mol | − 0.03 kcal/mol | − 5.12 kcal/mol | 10% | 394.532 |
| Isoniazid + 5KVU | − 4.50 kcal/mol | 500.64 µm | − 5.08 kcal/mol | − 0.07 kcal/mol | − 5.16 kcal/mol | 20% | 322.733 |
| Rifampicin + 1BVR | − 6.50 kcal/mol | 17.14 µm | − 6.63 kcal/mol | − 0.13 kcal/mol | − 6.76 kcal/mol | 20% | 760.267 |
| Rifampicin + 1UZN | − 7.89 kcal/mol | 1.63 µm | − 7.26 kcal/mol | − 0.14 kcal/mol | − 7.40 kcal/mol | 10% | 777.873 |
| Rifampicin + 3AF3 | − 6.96 kcal/mol | 7.97 µm | − 5.77 kcal/mol | − 0.76 kcal/mol | − 6.53 kcal/mol | 10% | 858.951 |
| Rifampicin + 5KVU | − 6.97 kcal/mol | 7.78 µm | − 5.63 kcal/mol | − 1.07 kcal/mol | − 6.71 kcal/mol | 10% | 1,001.869 |



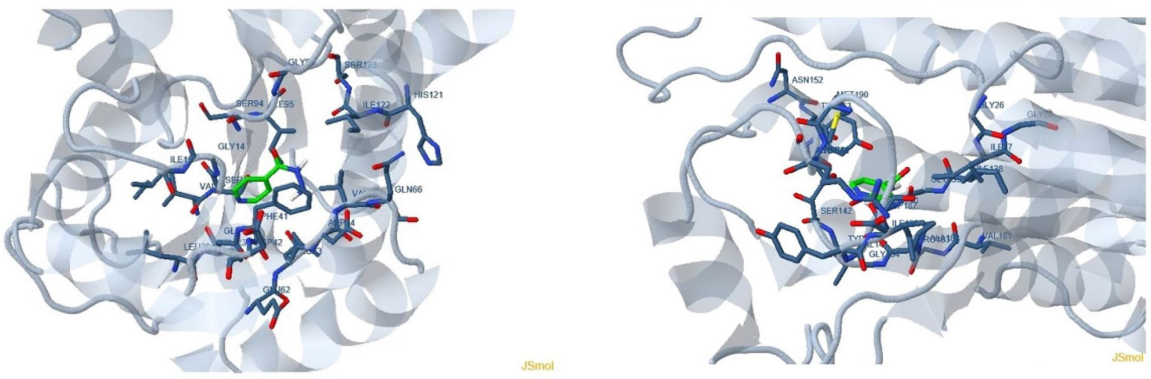
(a) (b)

**Figure 4. a and b:** (a) Molecular docking of astaxanthin and 1BVR— –Oxidoreductase. (b) Molecular docking of aAstaxanthin and 1UNZN— –Oxidoreductase. (c) Molecular of Astaxanthin and 3AF3–Transferase. (d) Molecular docking of Astaxanthin and 5KVU–Oxidoreductase.



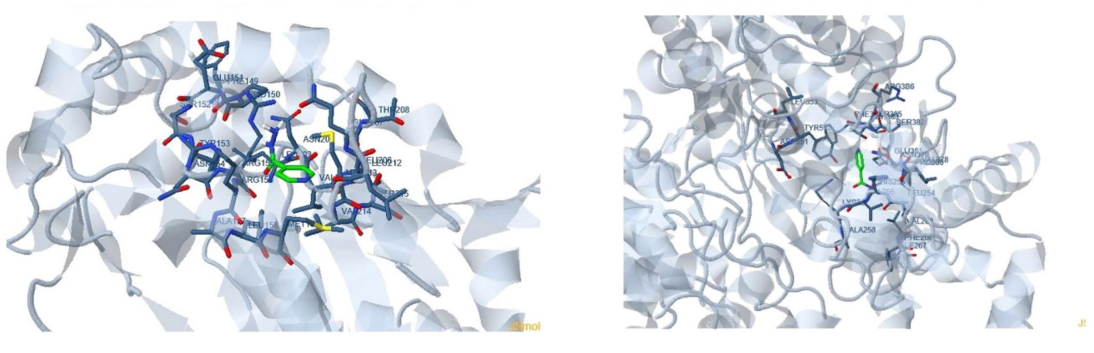
(c) (d)

**Figure 4.c and d:** a) Molecular of Astaxanthin and 3AF3— Transferase. d) Molecular docking of Astaxanthin and 5KVU— Oxidoreductase.



(a) (b)

**Figure 5. a and b:** (a) Molecular docking of Isoniazid and 1BVR— –Oxidoreductase. (b) Molecular docking of Isoniazid and 1UZN— –Oxidoreductase. (c) Molecular of docking of Isoniazid and 3AF3–Transferase. (d) Molecular docking of docking of Isoniazid and 5KVU–Transferase.



(c) (d)

**Figure 5.c and d:** a) Molecular of docking of Isoniazid and 3AF3— Transferase. d) Molecular docking of docking of Isoniazid and 5KVU— Transferase.

of 370.36, 659.54, 477.93, and 500.64 µm, respectively (Table 5). Molecular docking of isoniazid with PDB: 1BVR showed interaction through hydrogen bonds (N2 —THR39, N3 —LEU63), polar (H3 —ASP64), hydrophobic (C4 —ILE95, C1 —ILE95, C2 —ILE95), Pi-pi (C3 —PHE41) and cation-pi (H2 —PHE41, H3 —PHE41) as represented in Figure 5a. The amino acid residues N2 —GLU381, N2 —TYR552 (hydrogen bond), O1—LS257 (polar), and C6 —PHE384 (π–π) alone was involved in interaction between isoniazid and 5KVU (Fig. 5b). The other two protein such as 1UZN (Fig. 5c) and 3AF3 (Fig. 5d) bind with isoniazid by means of hydrogen bond, polar and hydrophobic interaction of different amino acid residues.

Rifampicin was docked with the binding site of the *Mycobacterium* proteins like IBVR, 1UZN, 3AF3, and 5KVU by auto dock of docking server. It was observed that rifampicin binds to the active-site of 1BVR by hydrogen bonds (N4 –TYR259), hydrophobic (C26 –ILE258, C21 –LEU250, C24 –LEU250, C30 –LEU250, C33 –LEU250, C40 –LEU250 and C43 –TYR259), and cation-pi (H6 –TRY259, H3 –TRY259) (Fig. 6a). The docking of rRifampicin with 1UZN showed the hydrophobic interaction with the amino acid residues like C25– TRP145, C38– TRP145, C43– TRP145, C17– TRP145, C41– TRP145, C12– TRP145, C14– TRP145, C20– ILE198, C7– PHE205, C12– PHE205, C30– PHE205, C33– PHE205, C40– PHE205, and cation-pi H5– PHE205[(Fig. 6b)]. Figure 6c illustrates the hydrogen bond formation with amino acid residues such as N3– PRO8, N11– PRO85, polar interaction with O4– GLN82, H4– ASP196 and hydrophobic of amino acid C26– ALA72, C7– PRO85, C30– PRO85, C33– PRO85, C40– PRO85, C11– PRO85, C31– PRO88, C43– HIS119 which was gained as a result of docking of rifampicin with 3AF3 protein. The binding interaction of rifampicin with 5KVU includes hydrogen bond formation with O10– TYR107, N4– TYR107, cation–pi of H6– TYR107 and polar interaction with O3– ASP43, H2– ASP43, O11– GLU61, H6– TYR107, O12– ASP722, H5– ASP722 which was shown in Figure 6d.

## 4. DISCUSSION

In many studies the anti-mycobacterial activity was confirmed by MIC determination methods, whereas, in present study, the activity was confirmed only by smear test and biochemical analysis. Similarly, anti-TB activity of *G. glabra* has only been reported with ethanolic extract against $H_{37}Ra$ and $H_{37}Rv$ strains (MIC = 500 µg/ml) of MTB [20]. Glabridin and Hispaglabridin B have also isolated from *G. glabra*. Glabridin exhibit the antitubercular

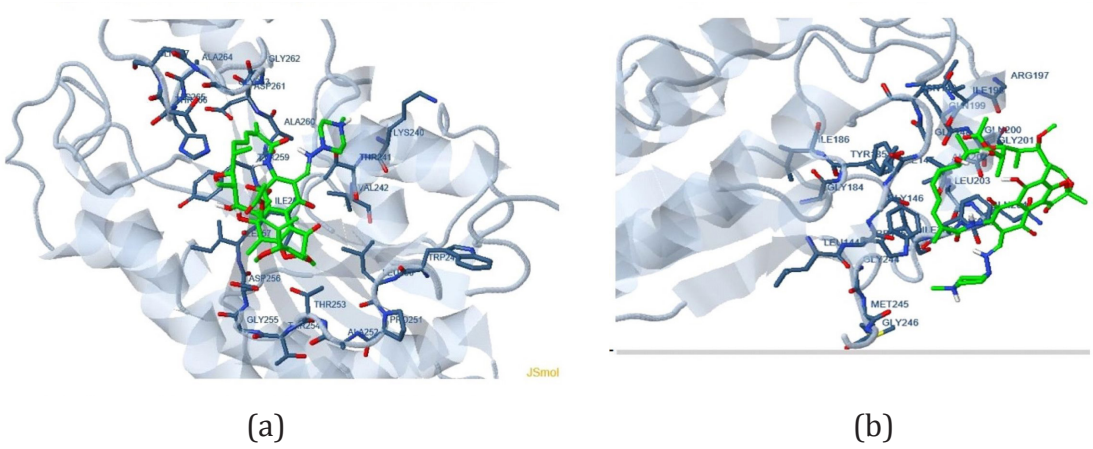



**Figure 6. a and b:** (a) Molecular docking of Rifampicin and 1BVR—– Oxidoreductase. (b) Molecular docking of Rifampicin and 1UZN— –Oxidoreductase. (c) Molecular of docking of Rifampicin and 3AF3–Transferase. (d) Molecular docking of docking of Rifampicin and 5KVU–Transferase.

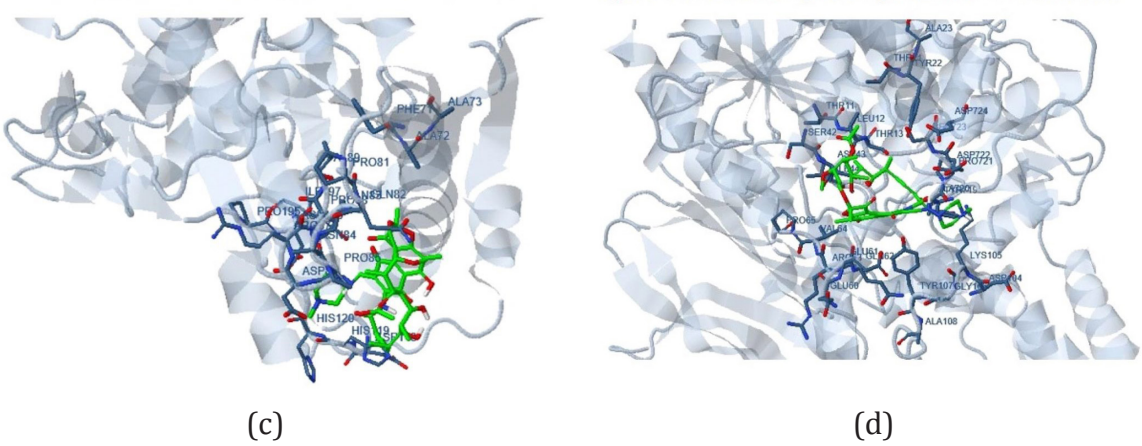



**Figure 6. c and d:** c) Molecular of docking of Rifampicin and 3AF3— Transferase. d) Molecular docking of docking of Rifampicin and 5KVU— Transferase.

activity and $IC_{50}$ was found to be at 29.16 µg/ml against both the strains ($H_{37}$Rv and $H_{37}$Ra). Vermelhotin, a natural tetramic acid from fungi, was also reported to be the most active drug toward clinical MDR TB isolates (MIC 1.5–12.5 µg/ml) [21].

For MTB and *M. bovis* strains, the MIC of 80% methanolic extracts of root of *C. aurea,* seeds of *O. basilicum,* leaves of *A. abyssinica, C. macrostachyus*, and *E. camaldulensis* showed 25–100 µg/ml and 12.5–75 µg/ml, 25–100 µg/ml and 25–50 µg/ml, 6.25–50 µg/ml and 12.5–50 µg/ml, 12.5–100 µg/ml and 18.25–50 µg/ml and 6.25–50 µg/ml and 12.5–50 µg/ml, respectively [22]. The presence of two ionone ring in carotenoids confers Vitamin-A like pharmacological activity. The actual role of ionone ring is not clear. It might be due to ozonolysis of ionone ring in the structure which results in formation of two geranic acid or due to the inhibition of matrix metallo proteins by Beta-Ionone. Thus, the molecular docking of astaxanthin towards various protein drug targets of Mycobacterium paved way towards search of new anti-TB drugs. Furthermore, the inefficiency of Astaxanthin to inhibit the growth of bacterial strains as shown by invitro studies explores the necessity of encapsulation. The microencapsulation of the drug improves the efficiency of drug towards inhibition of test strains. The structural basis for inhibition has been well demonstrated by molecular docking.

The bacterial DNA-dependent RNA synthesis was inhibited by rifampicin which inhibits the corresponding RNA polymerase [23]. Crystal structure and biochemical statistics propose that rRifampicin binds to the RNA polymerase (β subunit) within the DNA/RNA channel, but away from the active site [24]. Through this "steric-occlusion" mechanism, rifampicin breaks the second or third phosphodiester bond between the nucleotides in the RNA backbone and thereby preventing the elongation of the 5′ end of the RNA transcript which past more than 2 or 3 nucleotides [24,25]. The RNA synthesis was thus prevented by inhibitors at the elongation step and thus preventing the translation of RNA into host bacterial proteins.

Isoniazid is a prodrug which is activated by a bacterial catalase-peroxidase enzyme in MTB called KatG [26]. The formation of the isonicotinic acyl radical was catalyzed by KatG which spontaneously pair with NADH to form the nicotinoyl-NAD adduct. This complex binds tightly to the ACP reductase and thereby

blocking the natural enoyl-AcpM substrate and the action of fatty acid synthase. This process prevents the production of mycolic acids, which are the required components of the mycobacterial cell wall. A variety of radicals are synthesized by KatG including nitric oxide [27]. Isoniazid is bactericidal to rapidly breakdown mycobacteria but is bacteriostatic if the mycobacteria are growing slowly [28]. It inhibits the cytochrome $P_{450}$ system, and therefore acts as a source of free radicals. Isoniazid is a mild monoamine oxidase inhibitor [29].

## 5. CONCLUSION

In conclusion, the anti-mycobacterial activity of astaxanthin and liposome encapsulated Astaxanthin was evaluated to explore the pharmacological potential of microencapsulated pigment molecules as novel drug for TB. The *in silico* molecular docking analysis provided insights into mode of binding and interaction of the chosen drug with various anti-TB target proteins. As evidenced by smear test, astaxanthin can be proposed as novel anti-TB drug displaying potent binding with all the mycobacterial proteins when compared with two conventional anti-TB drugs (isoniazid and rifampicin). Furthermore, the research has to be focused towards *in vivo* testing to study the mechanism of action of selected lead compound in animal models.

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## 7. CONFLICT OF INTEREST

All the authors have equal contribution towards design, execution, completion of work, and framing of the manuscript, and hence no conflict of interest to be declared.

## 8. AUTHOR CONTRIBUTIONS

All authors made substantial contributions to conception and design, acquisition of data, or analysis and interpretation of data; took part in drafting the article or revising it critically for important intellectual content; agreed to submit to the current journal; gave final approval of the version to be published; and agree to be accountable for all aspects of the work. All the authors are eligible to be an author as per the international committee of medical journal editors (ICMJE) requirements/guidelines.

## 9. ETHICAL APPROVALS

This study does not involve experiments on animals or human subjects.

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