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ANTIOXIDANT ACTIVITY AND HPLC ANALYSIS OF THE MARINE ALGAE *HALYMENIA DILATA* AS A POTENTIAL FISH FEED FOR *POECILIA SPHENOPS*

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Abstract

Nowadays algal fishmeal is a principle source of protein in commercial aquatic feeds. Marine algae possess highly beneficial components that serve as a base and food for coastal fishes and aquatic organism. These marine algae are very important as they produce more oxygen. In the present investigation, the marine algae *Halymenia dilata* was collected from the coastal area and purified extract was prepared and optimized for the antioxidant activity and the presence of biologically active compound was analyzed by HPLC method. It was used as a potential fish feed for the ornamental fresh water fish *Poecilia sphenops*. The biochemical analysis and growth rate study further supported that the algae feed is better than the control feed.

Key words: Marine algae, *Halymenia dilata,* antioxidant, HPLC and *Poecilia sphenops*

Introduction

Ornamental fishes must be reared in high density in indoor systems as they can't scavenge freely on normal foods, and should be supplied with proper feeds. Complete diets should supply all the ingredients that are necessary for optimal growth and health of the fish (Altaff, 2015). Marine algae serve as a good source of protein for the fish. Low cost and good quality supplement feed provide essential nutrition and also a key demand for farmers to reduce the production cost. Due to the rising cost, uncertainty and unavailability of fish meal, it is an immense problem in modern aquaculture, especially in fish nutrition, to find a desirable replacement of fish meal. One of such international ornamental fishes is *Poecilia sphenops*. Despite its ornamental nature its normal needs and feeding pattern are not reported strongly, there is no detailed study on this fish variety so many researches have concentrated on detailed study of this fish (Lovell, 2000).

In this study black mollies (*Poecilia sphenops*) were reared in aquarium tanks and algal diet was formulated, that supported the growth and survival rate of the mollies. *Halymenia dilata* was selected as a potential fish feed since there is no study report on marine red algae so far. Fish feeds made up of algae are used for the ornamental fishes to maintain the quality of the fish and its health condition. To obtain a potential fish feed it is necessary to assess the antioxidant activity and the presence of active compound. Experiments were also conducted to study the growth and survival rate of the *Poecilia sphenops* using formulated algal diet.

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Materials and Methods

Sample Collection

Marine algae *Halymenia dilata* was collected from the coastal area. The collected sample was cleaned and dried then grinded into very well powder. The algal fish feed was prepared with suitable composition and was fed to the ornamental fishes *Poecilia sphenops* (Black Molly). In order to study the potential of fish feed the pure algae were subjected to Soxhlet extraction method and then the extract was evaporated. The remaining extract was stored for future use.

DPPH Assay

Screening for antioxidant activity was done by using DPPH assay. Based on the protocol described by Von

Gadow, the antioxidant effect of the algal extract was calculated. 2 mL of DPPH was mixed with sample and absorbance was read out immediately by using a spectrophotometer. We can observe the absorbance getting gradually reduced at 1 nm. Quercetin was taken as standard. The inhibition % was calculated from the absorbance (Von Gadow *et al.,* 1997). All observations were repeated in triplicate. Inhibition % was calculated through the following equation:

Inhibition % = $A_{Control}$- A_{Test}/$A_{Control}$× 100, where, $A_{Control}$ - Absorbance of control, A_{Test} – Absorbance of test sample

HPLC characterisation

The methanolic extract of the marine brown algae *Halymenia dilata* was characterized using HPLC (ZORBAX eclipse plus) with a photodiode array detector (PDA). The mobile phase used was milli-Q water (A) and acetonitrile (B) and 0.05 % of TFA (trifluroacetic acid) was also used. C18 column (4.6 x 250mm, 5ìm) was the stationary phase. HPLC grade solvents were used for the whole study. The gradient was set as per the procedure explained by Lawton and colleagues. (1994) and rate of flow was 1 ml per minute (Harada *et al.,* 1999). The injection volume was 20 µl, the temperature was maintained at 28ºC and separation was recorded at 275 nm.

Studies on growth and survival rate of *P. sphenops*

Experiments were conducted for 60 days to study the growth and survival rate of the *Poecilia sphenops* using formulated red algae and commercial fish feed. Initial weight and length of the fishes were noted. At the end of experiment, the length and weight of fish was noted. Water was periodically changed for the health of the fish. After 60 days of the treatment with control and algae feed, the fishes were sacrificed and the tissues were taken for the estimation of protein, lipids and carbohydrates.

Estimation of protein using Bradford reagent

Based on the dye binding technique, calculated the protein in tissue culture (Bradford, 1976). The reagents utilized for this experiment is coomassie brilliant blue (CBB) G-250 (100.0mg), 50 ml of ethanol (95%) and 100 ml of ortho-phosphoric acid (85%). The mixture of dye, ethanol and orthophosphoric acid were diluted with distilled water. Then the absorbance was measured at 9 nm by using a spectrophotometer and the solution mixture was filtered and kept in a bottle at 4°C. 1 ml of the culture filtrate was added with 5 ml of the Bradford's reagent (CBB) and the intensity of the blue color that developed was read at 595 nm in a spectrophotometer. The amount of protein was determined using bovine serum albumin fraction V (Sigma, USA) as the standard.

Estimation of total carbohydrate (Dubois 1956)

About 10 mg of extract was mixed in 10 ml of purified water. From this 1 ml was used for sugar analysis. To estimate the carbohydrate content in given extract, 1 ml of 5% phenol was added to 1 ml of plant extract solution, followed by 5 ml of concentrated H_2SO_4. The absorbance was measured after 10 minutes at 488 nm against blank (UV1800 UV-Vis Spectrophotometer, Shimadzu, Japan). Glucose served as standard.

Estimation of total lipid (Folch *et al.,* 1957)

About 100 mg of plant crude extract was extracted with 10 ml of chloroform: methanol (2:1) and solvent was separated and evaporated in vacuum. In brief, the standards and lipid samples were evaporated at 100°C. after that 0.1 ml of H_2SO_4 was added to each tube, and then heated for 10 minutes at 100°C. After that the tubes were cooled down and added 2.4 ml of vanillin reagent to each tube then vortexed. We could observe the development of pink colour after that added 0.2 ml of samples and standards to each tubes. The absorbance was measured at 490 nm (UV1800, UV-Spectrophotometer, Shimadzu, Japan.

***In silico* molecular docking**

The docking calculation was performed for protein with ligand (bioactive compound) and the target receptor Human Cytochrome P450 protein. It was done successfully using iGEMDOCK. It is a graphical representation of known therapeutical relations and search the structure of suitable small molecules to bind to a drug target. iGEMDOCK provide physiological factors of a living organism and discover the pharmaceutical factors from chemical compounds. The pharmaceutical properties of the sample represent preserved interrelated molecules that frequently form binding sites with unique physical and chemical factors to perform the significant functions of the target protein. The final output represent that the hit rate of iGEMDOCK is around 78%.

Results and Discussion

Anti-oxidant activity of the marine algae *H. dilata*

The marine macro algae *Halymenia dilata* were used for evaluation of antioxidant effect by diphenyl picrylhydrazyl assay method, various concentration of extract was checked. Methanol was taken as standard and the absorbance was read out, it changed with the concentration. The Control was methanol and the OD was 0.614 constant with all the concentration and the

OD of the sample varied with the concentration.

Seaweed is the big supply of antioxidants. Because of that different varieties of seaweeds were studied to recognize novel and efficient antioxidant composites, and also explain the method of cell propagation and cell death. (Siriwardhana *et al*., 2004; Athukorala *et al*., 2005; Heo *et al*., 2005; Park *et al*., 2005).

Many researchers have found seaweeds to be a rich source of antioxidants. Antioxidants play major role against many diseases. Ascorbate and Gluetathion are reactive antioxidant molecules which are present in fresh as well as secondary metabolites including carotenoids mycoporine (Vadlapudi *et al*., 2012). Generally marine algae has compounds that has chemical defense system facilitating their survival. Antioxidant are compounds that inhibit oxidation. Marine macroalgae has anticancer, antimicrobial, antifungal and antioxidant activities (Ashwini *et al.,* 2017).

From our results it is predicted that antioxidant activity of *Halymenia dilata* was higher in 50 µL concentration with 82.24 %. DPPH assay results are shown in the Fig. 1.

HPLC characterization of compounds produced by *Halymenia dilata*

HPLC is an exact form of column chromatography that was performed in *Halymenia dilata* to divide, recognize and enumerate the lively particles. HPLC helps in identifying the number of compounds present in it. It also includes the procedure of separation and decontamination of particles. Fig. 2 shows the HPLC chromatographic pattern at 275 nm and six peaks were identified with a retention time (RT) of 3.299 min, 3.825 min, 4.283 min, 9.513, 11.889 and 12.606 min table 1. The maximum peak was obtained with a retention time of 11.889 min with the absorbance of 135.51 mAU.

Estimation of Protein Content

Proteins are the very luxurious division of fish supply,

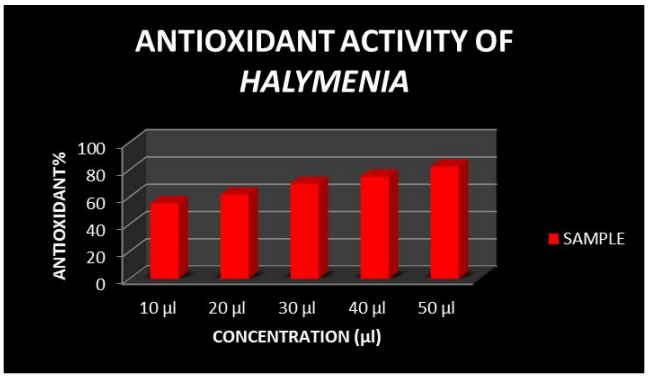

Fig. 1: Antioxidant activity of *Halymenia dilata* extracted in methanol solvent.

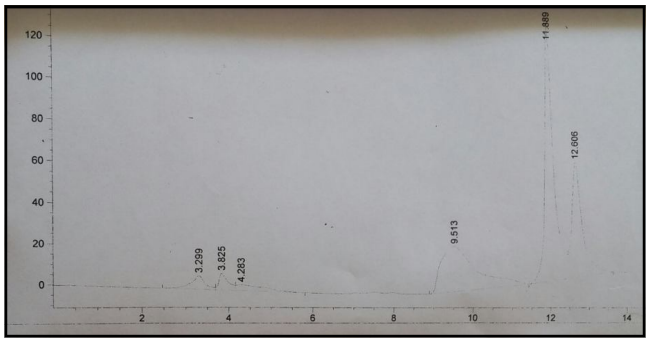

Fig. 2: HPLC chromatogram with the peak at maximum RT at 11.889 min at 275 nm.

Table 1: HPLC peaks with the retention time of compounds identified from methanolic extract of *Halymenia dilata*.

peaks	RT (min)	Width (min)	Area (mAU*s)	Height (mAU)	Area (%)
1	3.299	0.3046	150.40	6.70	2.88
2	3.825	0.2103	122.61	8.30	2.35
3	4.283	0.6046	134.71	2.84	2.58
4	9.513	0.8488	1455.70	23.59	27.95
5	11.889	0.2290	2171.68	135.51	41.70
6	12.606	0.2723	1171.82	61.22	22.50

because of that it's very significant to exactly establish the proteins supplies for every type. Protein rate affect the weight of fish in different types (Chong *et al*., 2004). Protein necessities differ with atmosphere, water feature, genetic work and feeding rates of the fish.

The protein content in muscle, liver and the gills of the fish in control feed were 2.81mg/g, 0.75mg/g, 2.07 mg/g and the protein content in the muscle, liver and gills of the fish in algae feed were 1.8mg/g, 1.215mg/g, 2.675mg/g respectively. The protein content in muscle of control feed was higher compared to algal feed. The protein content of the liver and gills of the algal treated fish was higher compared to the fish treated in control feed as in Fig. 3.

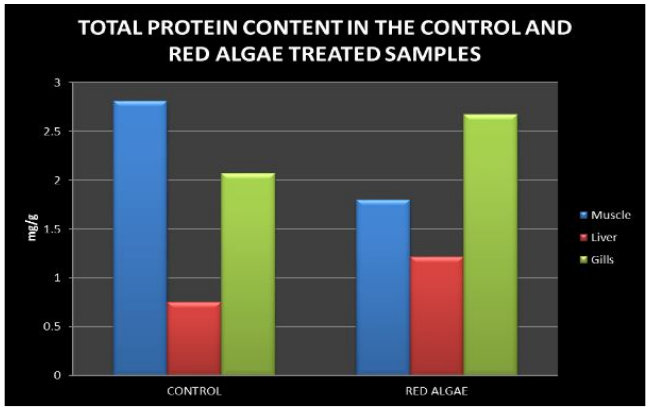

Fig. 3: Total proteins content in the control and red algae treated fish samples.

Estimation of Lipid Content

The lipid content in the muscle, liver and gills of the fish in control feed were 1.4624mg/g, 0.0824mg/g, 1.104 mg/g and the lipid content in the muscle, liver and gills of the fish in algae feed were 0.7674mg/g, 0.39mg/g, 2.2525mg/g. The lipid content of the liver and gills in the algae treated fish was higher compared to the control feed. Simultaneously the lipid content in muscle of the control feed was higher in comparison to algae feed as in Fig. 4.

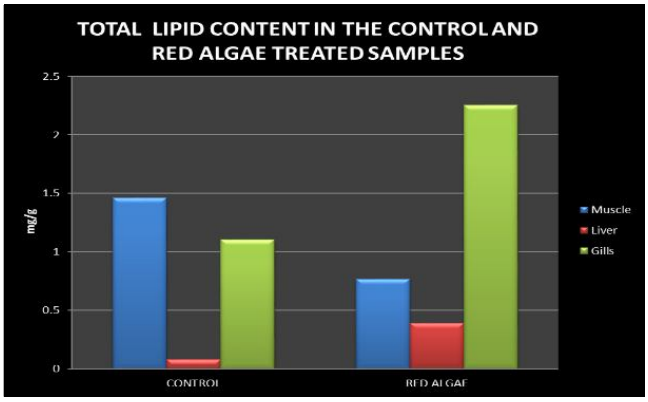

Fig. 4: Total lipids content in the control and red algae treated fish samples.

Estimation of Carbohydrate Content

Carbohydrates are the popular energy supplement in the fish foods, because it is cost-effective. The important pathways of carbohydrate metabolism are cytoplasmic pathway, citric acid cycle, phosphogluconate pathway, metabolic pathway, and Glycogenesis, have been explained (Shimeno, 1974).

The carbohydrate content in the fish muscle, gills and liver in control were 1.1714mg/g, 0.377mg/g, 0.6286 mg/g and in algal feed were 0.4084mg/g, 0.36mg/g, 0.337mg/g. Hence the carbohydrate content in muscle was found to be higher in control feed compared to the algae feed. There is no much difference seen in the carbohydrate content in the liver and gills of the fish in both control and red algae feed as seen in Fig. 5.

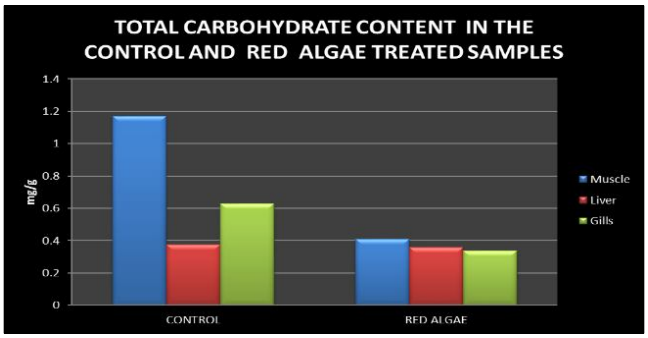

Fig. 5: Total carbohydrates content in the control and red algae treated fish samples.

Growth and survival rate of the fish

The fishes fed with red algae feed showed more growth and the fishes were seemed to be more active than the fishes in the control feed. The weight of fish of the algae feed were 2 g higher than the control feed Fig. 6. The length of fishes in control initial length was 5.04±0.55cm and final length was 5.95±0.66cm whereas in algal feed initial length was 5.02±0.85cm and final length was 6.05±0.11cm Fig. 7. The survival rate of fish in red algae was found to be 99.99% and whereas in control it was only 33.32% Fig. 8. The mortality rate in red algae is 0.05% whereasin control it was 66.67 % Fig. 9.

The findings of the present study supports, that the fish feed prepared from the marine red algae *Halymenia dilatata* serve as a good nutritional and also proves to be a rich source of antioxidant for ornamental fish species *Poecilia sphenops* (Black molly). The current study focused on the preparation of marine red algae fish feed as a replacement for the commercial feed to the fishes. By applying these marine algae feeds, ornamental fish

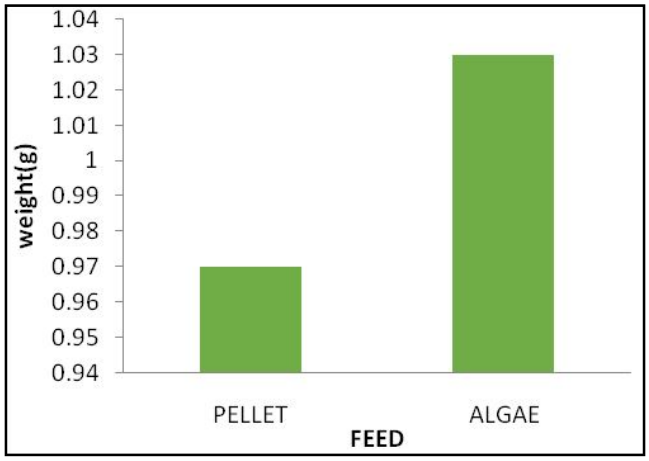

Fig. 6: Total weight gain of the fish in control and red algae feed after 60 days.

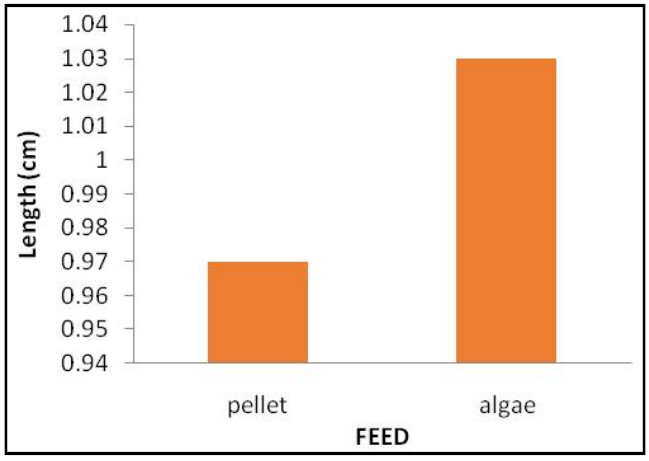

Fig. 7: Total length gain in fish of control and algae feed after 60 days.

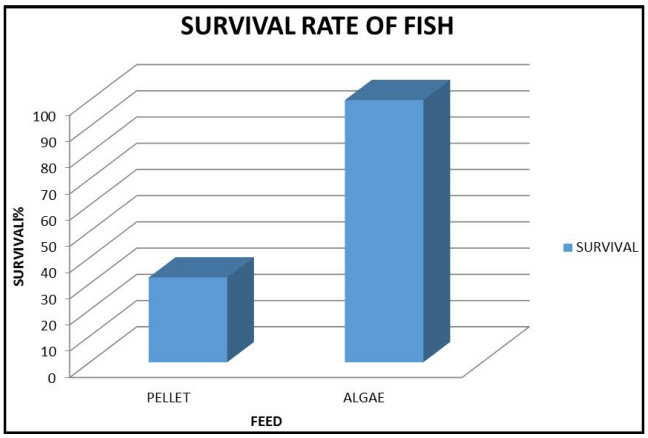

Fig. 8: Survival rate of the fish in control and algae feed.

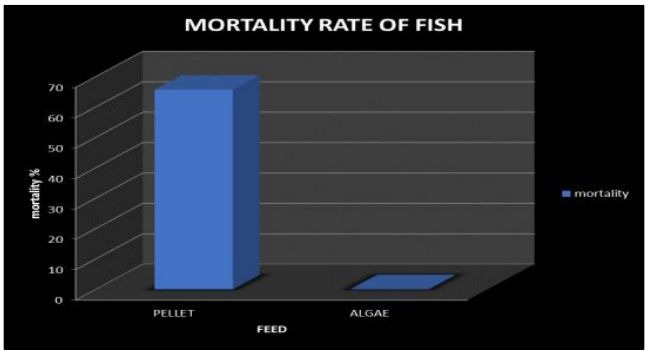

Fig. 9: Mortality rate of the fish in control and algae feed.

Fig. 10: Production of fries (23) in female molly fed with algae feed.

farming becomes more profitable and cheaper by lowering the feed cost. The nutrient content of protein and fat in *Poecilia sphenops* is higher in the marine algae feed

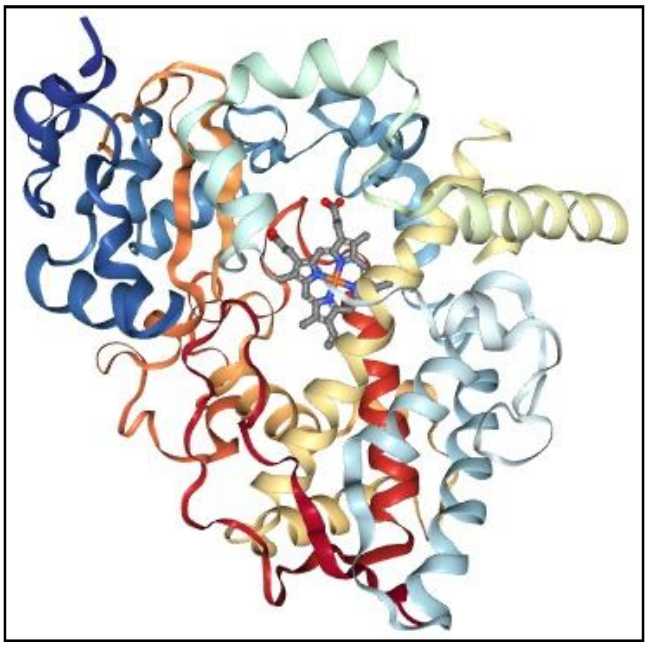

Fig. 11: Structure of Human Cytochrome P450 protein (PDB ID: 1W0E).

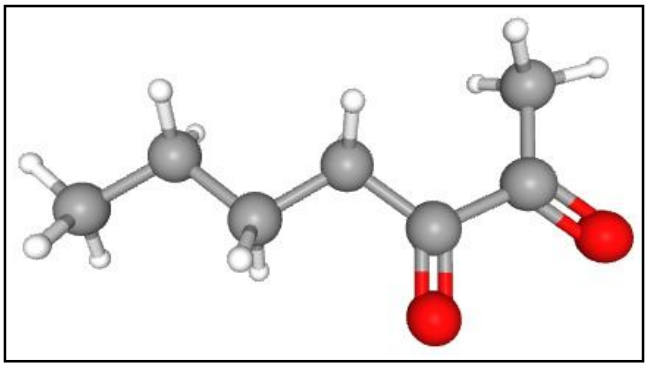

Fig. 12: Structure of Acetyl Valeryl (PubChem CID:60983).

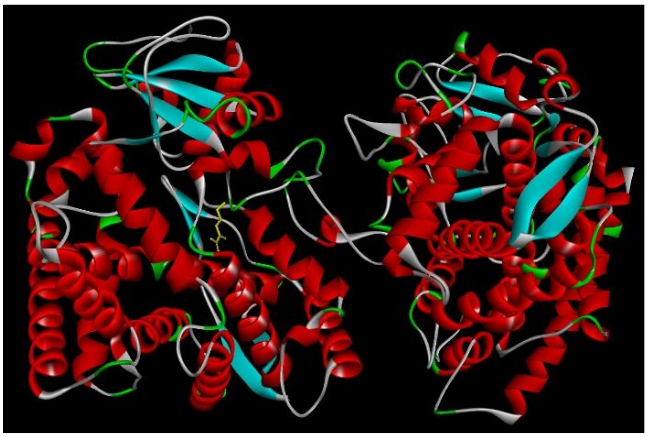

Fig. 13: Acetyl valeryl bound with human Cytochrome P450.

Table 2: Molecular docking result with the ligand and the fitness values.

Ligand	Total Energy	vdW	Hbond	Elec	Aver ConPair
Acetyl Valeryl	-63.071	-36.449	-26.622	0	21.65

than the commercial feed The female fish fed with algae feed after 60 days have also produced 23 fry at a time and is shown in Fig. 10.

Molecular docking interaction analysis

Bioinformatic modelling was effectively done among preferred molecule (Acetyl valeryl) and target receptor cytochrome P450 protein. The total energy of a predicted pose in the binding site is the fitness value. The empirical scoring function of iGEMDOCK was estimated as;

Fitness = vdW + Hbond + Elec.

Here, vdW is the van der Wall energy, Hbond and Elect are hydrogen bonding energy and rank.

The table 2 shows the ligand acetyl valeryl and its fitness values and the structure of cytochrome P450, Acetyl valeryl and the *in silico* interaction between cytochrome P450 and the ligand Acetyl valeryl were depicted in the Fig. 11, 12 and 13 respectively. Powerful binding shows extra positive energies, without binding possess negative energies (Krieger and Vriend, 2014). Bioinformatic modelling results among Acetyl valeryl and cytochrome P450 showed weak strength of the binding interaction with negative energies for particular substances.

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