IJBPAS, October, 2020, 9(10): 2594-2605

ISSN: 2277–4998

International Journal of Biology, Pharmacy and Allied Sciences (IJBPAS)

'A Bridge Between Laboratory and Reader'

www.ijbpas.com

DETERMINATION OF BINDING AFFINITY OF FLAVONOIDS EXTRACTED FROM TRADITIONAL SPICES ON HISTIDINE DECARBOXYLASE

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Received 6th Feb. 2020; Revised 3rd March 2020; Accepted 6th April 2020; Available online 1st Oct. 2020

https://doi.org/10.31032/IJBPAS/2020/9.10.5208

ABSTRACT

The enzyme histidine decarboxylase catalyses the conversion of amino acid histidine to histamine. Hypersensitivity is an undesirable reaction produced by the host immune system in response to certain environmental agents. Histamine acts as a mediator and brings about allergic responses due to mast cell degranulation. Generally, antihistamines are used to treat allergic reactions and hypersensitivity. But antihistamines have various side effects associated with it. Therefore, the aim of the study was to find an alternative to these antihistamines by inhibiting histidine decarboxylase. The flavonoids present in spice extracts are responsible for this enzyme inhibition. Molecular docking was performed with the isolated flavonoids to predict its interaction with the target, i.e., histidine decarboxylase using the web-based software 1-Click docking. It is necessary to have a good binding affinity for the ligand to form a stable complex with its target in physiological conditions. The greater the negative value, the higher is the binding of the ligand to its target. The highest binding efficiency of Gallic acid, Ellagic acid, Kaempferol and Quercetin was found to be -5.8, -7.9, -7.3 and -7.2 respectively. Ellagic acid and Kaempferol proved to be the most promising molecules in comparison to all the other isolated flavonoids.

Keywords: Molecular docking, histidine decarboxylase, gallic acid, ellagic acid, quercetin, kaempferol

INTRODUCTION

Histidine decarboxylase converts the aromatic amino acid histidine, to histamine by a PLP-mediated decarb-oxylation. Histamine has a series of physiological applications from being a neurotransmitter in the neurons of the brain to controlling the release of gastric acid by the enterochromaffin cells. One major role of histamine is in type I hypersensitivity or anaphylaxis where it causes an inappropriate immune response.

Enterobacter species and Lactococcus species were isolated from garden soil and curd respectively and characterized in order to identify them. These were used as sources of the enzyme, histidine decarboxylase. The enzyme was isolated by sonication and purified by ammonium sulphate precipitation, ion-exchange chromatography and molecular exclusion chromatography.

Traditional spices such as clove, fennel and cardamom are a potential source of natural antioxidants. One of the major phytoconstituents of spices are flavonoids which are majorly responsible for its biological activity. The flavonoids were extracted from the methanolic extracts of spices and purified by using TLC and HPLC.

Currently, antihistamines are used to treat allergies but produce a range of side effects which can even be lethal. An alternate to antihistamines can be bought into existence by inhibiting the enzyme responsible for the production of histamine. The present study aims at inhibiting the enzyme histidine decarboxylase using flavonoids extracted from the spice. Further in silica docking was used to determine the binding affinity of flavonoids to the enzyme.

MATERIALS AND METHODS

Chemicals: Acacia powder, acetone, ammonium persulphate, benzene, bis-acrylamide, boric acid, bromophenol blue, chloroform, citric acid, crystal violet, di-sodium hydrogen phosphate, disodium tetraborate, ethyl acetate, EDTA (Ethylene diamine tetraacetic acid), ferric chloride, glacial acetic acid, Gram's Iodine, hydrochloric acid, hydrogen peroxide, liquor ammonia, methanol, nitric acid, oxalic acid, potassium hydroxide, potassium iodide, potassium sodium tartrate, safranin, sodium acetate, sodium carbonate, sodium chloride, sodium dihydrogen orthophosphate, sodium hydrogen carbonate, sodium lauryl sulphate, sulfuric acid, trisodium citrate were procured from **Fisher scientific**. Acrylamide, agar powder, β mercaptoethanol, Coomassie Brilliant Blue, eosin methylene blue agar, glycerol,

glycine, histidine, Kovac's indole reagent, Mac Conkey agar, MRS (De Man, Rogosa and Sharpe) broth, MR-VP broth, nutrient agar, nutrient broth, peptone, potassium acetate, quercetin, Simmon's citrate agar, sodium hydroxide, TEMED (N, N, N', N' – tetramethyl ethylenediamine), tris free base, Wuster's reagent (N, N, N', N' – tetramethyl-p-phenylenediamine dihydrochloride) were procured from **Hi-Media**. Alizarine red S was procured from **Chemie Pvt Ltd**. Mercuric chloride, methyl red was procured from **Merck**. Cedarwood oil, Dragendroff's reagent, tryptone were procured from **Nice Chemicals**. n – butanol was procured from **Qualigens by Thermofisher**. Aluminium chloride, Ammonium sulphate, copper sulphate, histamine, nickel sulphate heptahydrate, ninhydrin was procured from **S D Fine chem Ltd**. Carboxymethyl sepharose, diethyl aminoethyl sepharose, quercetin, Sephadex G-100 were procured from **Sigma Aldrich**. α- naphthol was procured from **Thomas Baker**.

Isolation and culturing of bacteria – Enterobacter and Lactococcus were isolated from garden soil and freshly fermented curd respectively. Serial dilutions including agar plating were employed to isolate the bacterial culture from the source followed by subculturing on MRS **[1]**, Mac Conkey agar **[2]** and Eosin methylene blue agar **[3]**.

Characterization of bacteria - The bacteria was characterized by performing Biochemical tests such as IMViC test **[4, 5]**, Catalase test **[6]**, Oxidase test **[7]** and Gram staining **[8]**.

Extraction and Partial Purification of enzyme – Extraction of enzyme was done by sonication of culture at a pulse rate of 0.5S and amplitude of 40% for 30 minutes, maintained at 25ºC following partial purification by 30%, 60% and 80% ammonium sulphate precipitation **[9]**.

Purification of the enzyme by CM – sepharose Ion exchange chromatography - The CM – sepharose was activated using 50 mM, acetate buffer pH 5 and was packed into column (10cm×1.6cm) at room temperature and the column was equilibrated with start buffer at a flow rate of 25 ml/hr. The 30 % ammonium sulphate precipitated fraction was loaded onto the column and the column washed with two-bed volumes of start buffer. The bound proteins were eluted with a stepwise increase in the ionic strength (0.1M, 0.3 M and 0.5 M NaCl). Fractions of 5 ml were collected. The enzyme histidine decarboxylase did not bind to the column and was eluted in the washings (CMS – I fraction). This fraction was further subjected to anion exchange chromatography in the DEAE – sepharose column **[9]**.

Purification of the enzyme by DEAE – sepharose Ion exchange chromatography - The DEAE – sepharose was activated with 50 mM, phosphate buffer pH 7 and was packed into column (10cm×1.6cm) at room temperature and the column was equilibrated with two start buffers at a flow rate of 25 ml/hr. The CMS - I fraction was loaded onto the column and the column was washed with two-bed volumes of start buffer. The bound proteins were eluted with a stepwise increase in the ionic strength (0.1M, 0.3 M and 0.5 M NaCl). Fractions of 5 ml were collected. The enzyme histidine decarboxylase was bound to the column and was eluted in the washings (DEAE S – VI fraction). This fraction was further subjected to gel permeation chromatography in Sephadex G-100 column **[9]**.

Purification of the enzyme by Molecular exclusion chromatography – The DEAE S – VI fraction was filtered through Sephadex G-100 column (20cm×0.8cm). Sephadex G – 100 gel was equilibrated with 10 mM sodium acetate buffer, pH 5 and packed into a column (200mm×8mm). The column was then equilibrated with two-bed volumes of 10 mM sodium acetate buffer, pH 5 at a flow rate of 10 ml/hr. The DEAES fraction, DEAES-VI containing histidine decarboxylase activity was subjected to gel permeation chromatography separately using Sephadex G – 100. The proteins were eluted with start buffer and fractions of 2 ml were collected **[9]**.

In-gel assay of enzyme – The samples of the enzyme was run on Separating gel with 10% polyacrylamide at 50V for 1-1.5 hours. After electrophoresis, the gel was made to react with 1mM substrate, histidine and incubated. After incubation, the gel was treated with Alizarine Red S, Nickel Sulphate and Acetate buffer in order to observe the bands.

Determination of optimum pH – Effect of pH and various buffers on HDC activity was determined by incubating and carrying out the reaction at various pH ranging from pH3 to pH9 by pre incubating the enzymes with different buffers including Citrate buffer, Carbonate Buffer, Phosphate buffer, Acetate buffer and Borate buffer **[10]**.

Determination of thermostability and optimum temperature - The thermal stability of the enzyme was determined by pre-incubating the enzyme fraction at various temperatures between 0˚C - 70˚C and then checking the enzyme for its activity **[10]** and the optimum temperature was determined by conducting the enzyme assays at temperatures ranging from 25˚C-50˚C **[10]**.

Determination of K_m and V_{max} – The kinetic constants of HDC was determined

by incubating fixed amount of the enzyme with varying concentrations of the substrate, histidine by constructing LB-plot, Michaelis Menton plot, Eadie Hofstee plot and Hanes-Woolf plot **[11]**.

Plant sample preparation and phytochemical analysis – Random sampling was done by collecting Fennel seeds, Green cardamom and clove and 20% methanol extract of the sample was prepared with 70% methanol followed by centrifugation to collect the supernatant for further experiment.

Quantification of flavonoids - 0.2-1.0mL aliquots of the standard quercetin solution and made up to 2mL with methanol in each tube. 2.0 mL of methanol was taken as blank. To this reaction mixture, 0.1mL of 10% $AlCl_3$ was added followed by 0.1mL of 1M potassium acetate and 2.8mL of distilled water. The tubes were then incubated for 30 minutes at room temperature and the absorbance was read at 415nm at the end of the incubation period. The flavonoid content was measured as mg quercetin equivalent/g of the extract **[12]**.

Purification of flavonoids – Flavonoids were purified by TLC using n-butane: ethyl acetate: distilled water (5:10:15) as the solvent system and mixture of 3% boric acid and 10% oxalic acid as the spraying agent to detect the spots and by HPLC system (Agilent Technologies Company) with dual lamp binary system, UV detector, C18 column (i.e., 4.6 mm×150mm, 5µm) and data was integrated by Agilent Chem Station software. Standards and sample extracts were analyzed using the following gradient program (A.100% acetonitrile B HPLC Grade Water 0min, 5%A: 10min, 15% A: 20 min, 25% A: 30min, 35%A: 40min, 45%A: 50min 55% A). The flow rate was 0.8 mL/min and injection volume were 20µL. Detection was done at 280 nm **[13]**.

Inhibition of histidine decarboxylase – The enzyme was allowed to interact with the flavonoids present in the spice extracts. It was then allowed to catalyze the decarboxylation of histamine and its activity was determined. The results were then inferred in order to identify the type of inhibition by varying the concentrations of the enzyme and the substrate keeping the concentration of the inhibitor constant.

Determination of Binding affinity of inhibitors to the enzyme - The binding affinity was determined by 1-Click docking with the different flavonoids as ligands against the enzyme as the target.

RESULTS AND DISCUSSION

Histidine decarboxylase is a biologically significant enzyme as the product produced by its catalysis i.e., histamine plays a variety of physiological roles. One major role is its importance in anaphylaxis wherein it acts as a modulator in eliciting

the immune response thus, causing an allergy. Blockers of the histamine receptors are the present-day antihistamines which cause a range of after-effects. Reduced production of histamine can alter the intensity of allergy which can be achieved by partial inhibition of histidine decarboxylase.

Isolation and purification of Histidine decarboxylase: The Enterobacter spp was identified using the biochemical tests and gram staining **(Table 1)**. The identified organisms were isolated and subjected to large scale culturing in order to obtain greater biomass. This culture was subjected to sonication to obtain cell-free extract from which the enzyme, histidine decarboxylase was purified by ammonium sulphate precipitation, ion-exchange chromatography and molecular exclusion chromatography. The total activity, total protein, specific activity, fold purity and percentage yield of the enzyme was checked at every step of the purification process which has been listed in **Table 2**. Lastly, in order to confirm the enzyme purity, the samples from various steps of purification were loaded on an SDS-PAGE and a zymogram was developed by staining the gel with Alizarine Red S and Nickel Sulphate Heptahydrate post treatment of the gel with the substrate, histidine for a time span of 30 minutes. The presence of the bands in the samples obtained from anion exchange chromatography and molecular exclusion chromatography indicates the presence of the purified enzyme **(Figure 1)**.

Properties of histidine decarboxylase – The purified enzyme was used to study the chemical properties. The thermostability and the pH stability of the enzyme along with the other characteristics were determined. It was observed that the enzyme exhibited thermostability over a range of temperatures between 25°C to 55°C. The pH stability of the enzyme was studied in the presence of various buffers providing a range of pH. The buffer systems used included the citrate buffer system, the phosphate buffer system, the borate buffer system and the carbonate buffer system. Of all these buffer systems, the enzyme was found to exhibit great activity in the presence of the citrate buffer system and showed stability around the pH range of 5 to 7. The other properties of histidine decarboxylase are listed in **Table 3 and Table 4**.

Purification of flavonoids - The flavonoids were detected using a mixture of 3% boric acid and 10% oxalic acids. The spots were observed as green and blue fluorescence when viewed under the short wavelength of UV light using an UV-transilluminator depending on their

structure. The samples were subjected to HPLC in the acetonitrile: HPLC grade water system with standards of gallic acid, ellagic acid, kaempferol and quercetin as a reference in order to identify the flavonoids present in our sample using the retention time. The flavonoids identified by comparing the retention time of the peaks with that of the standard compounds using HPLC indicated the presence of Gallic acid, ellagic acid, quercetin and kaempferol in the extracts **(Figure 2, 3)**.

Inhibition of histidine decarboxylase - The methanolic extracts of fennel and clove were used to study the enzyme activity and a marked decrease in the activity was observed with increasing concentration of the extract. The inhibitory concentration of the clove and fennel was found to 0.9 and 0.625 µmoles respectively **(Figure 4, 5)**.

Determination of Pharmacokinetic and docking properties - It is a basic necessity of any hit molecule or lead compound to pass a certain set of criteria in order to be a potential drug molecule. The Lipinski's rules are a set of basic rules to be met by any lead compound. All the components identified in the HPLC quantification such as gallic acid, ellagic acid, quercetin and kaempferol follow the basic set of Lipinski's rules. Gallic acid and Ellagic acid have very low bioactivity scores indicating their ability to qualify as potential drug molecules are comparatively low. On the other hand, quercetin and kaempferol are promising compounds with a good bioactivity score but have chances of cross-reacting with other receptors which need to be tackled by modifying the side chains of the molecule. For a drug molecule to be effective it is very necessary that they are absorbed into the bloodstream from the site of admistration and also they need to be excreted without causing harm to the host system. Absorption plays a major role as it is the root of all the other processes to occur. These compounds are very promising with the absorption through the GI tract but it has to be kept in mind that absorption through GI tract can pave way for first-pass metabolism which can alter the molecule even before it reaches its target. The lipophilicity coefficient values indicate that the molecules can enter the cell through diffusion but need to be carried by another molecule through the bloodstream as they are comparatively less water-soluble. They don't inhibit the liver cytochrome enzymes and thus, the process of biotransformation will not be much affected and liver toxicity wouldn't occur. They all lack the ability to cross the blood-brain barrier thus, the nervous system is safe and unaffected. Molecular docking is a computer-based tool to check the interactions of the ligand with its target. In

case of possible interaction, it is very necessary that they have a good binding affinity to form a stable complex in physiological conditions. The greater the negative value, the higher is the binding of the ligand to its target. The binding affinities are represented as kcal/mol and are a very good way to describe the pose at which the ligands have an affinity to the protein. Gallic acid was the least promising molecule as it gave binding affinities within -6. Ellagic acid was a very promising molecule as it showed a range of binding affinity from -6.0 to -7.9. It exhibits a strong binding with the target and can be a potential lead compound. Quercetin is a less promising compound compared to ellagic acid but a better compound compared to Gallic acid. Better values are observed for binding efficiency in comparison to gallic acid. It showed binding affinity ranging from -6.2 to -7.2. Kaempferol is the best of all the compounds studies and the most promising one. Though the location for binding to the target is limited, the efficiency of binding is really high. The binding affinity values ranged from -7.1 to -7.3. The binding affinities vary largely due to the residues present at the enzyme pockets. HDC is said to have 6 pockets of which the largest pocket situated right at the centre of the enzyme provides the greatest binding affinity. The residues that interacted the most with the flavonoids components were found to be Serine, Glutamate, Aspartate and valine residues **(Figure 6-9)**.

Table 1: Biochemical Tests and Gram staining of bacteria

Biochemical Tests	Soil Sample
Indole Test	Negative
Methyl red Test	Negative
Voges Proskauer Test	Positive
Citrate Test	Positive
Catalase Test	Positive
Oxidase Test	Positive
Gram staining	Gram negative Bacilli

Table 2: The activity of HDC in case of Enterobacter at various steps of purification

Enzyme sample	Total Activity (IU)	Protein (mg)	Specific activity (IU/mg)	Fold Purity	Percentage Yield
Crude enzyme	42.476	567	0.07	1	100
30% Ammonium sulphate precipitation	0.011125	37.7475	0.0002947	0.00421	0.0261
Cation Exchange Chromatography	0.1019	51.187	0.00197	0.0281	0.239
Anion Exchange Chromatography	3.08875	154.087	0.02004	0.286	7.27
Gel Permeation Chromatography	12.430	120.815	0.10288	1.469	29.26

Lane 1 - Gel permeation Sample
Lane 2 – Anion exchange sample
Lane 3 – Cation exchange sample
Lane 4 –30% ammonium sulphate sample
Lane 5 – Crude enzyme

Figure 1: Zymogram of Histidine Decarboxylase activity

Table 3: The chemical properties of histidine decarboxylase

Chemical Properties	Obtained results
V_{max}	0.0012 IU
K_m	0.588 mM
Optimum pH	7
Optimum temperature	50°C

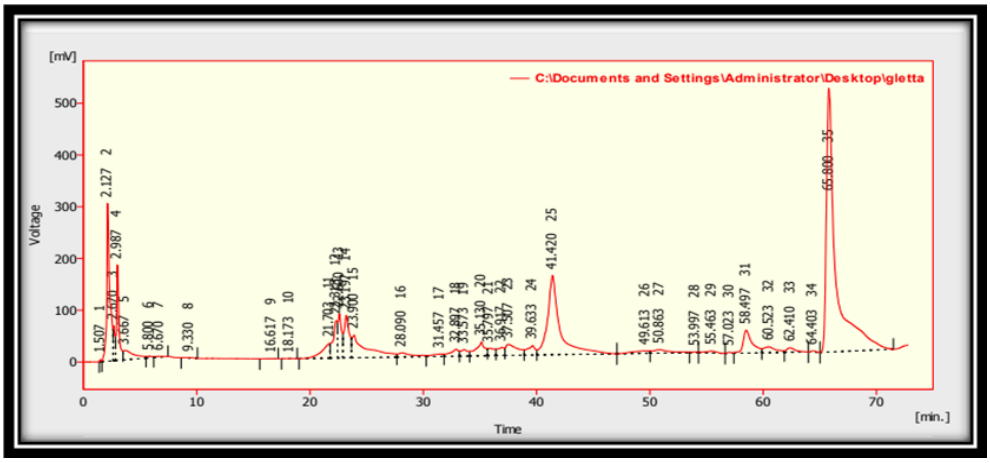

Figure 2: HPLC of Clove sample

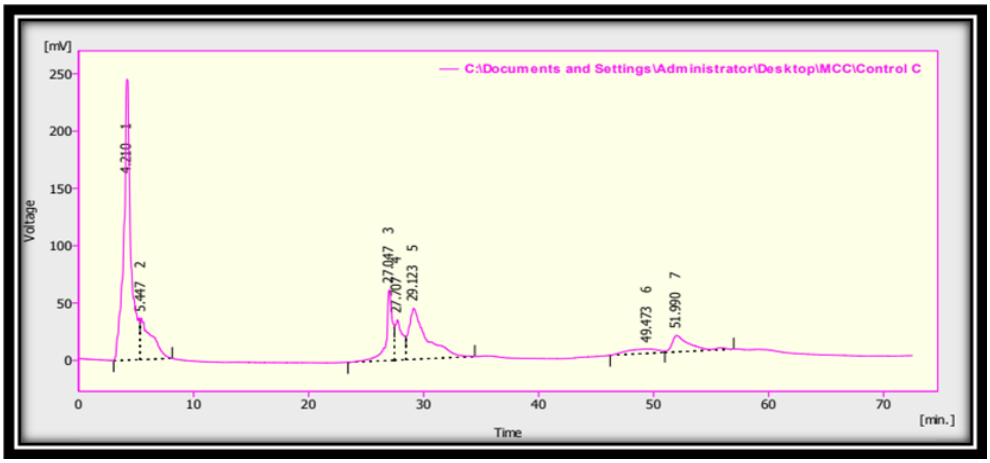

Figure 3: HPLC of Fennel sample

Table 4: Determination of K_m and V_{max} values of HDC

Name of the plot	K_m (Mm)	V_{max} (IU)
Michaelis Menton Plot	0.55	0.00106
Lineweaver Burk Plot	0.588	0.0012
Eadie Hoftsee Plot	0.663	0.0011
Hanes Woolf Plot	0.35	1.03

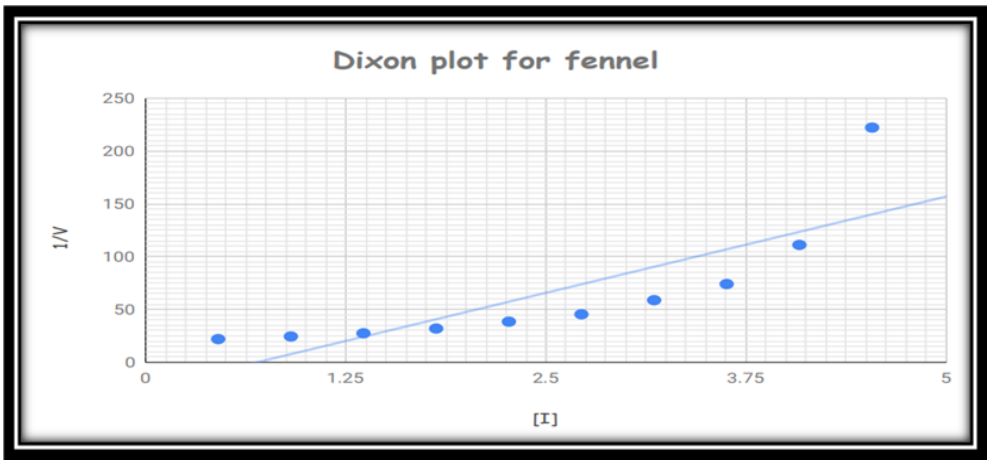

Figure 4: Dixon plot determining the inhibitory concentration of fennel

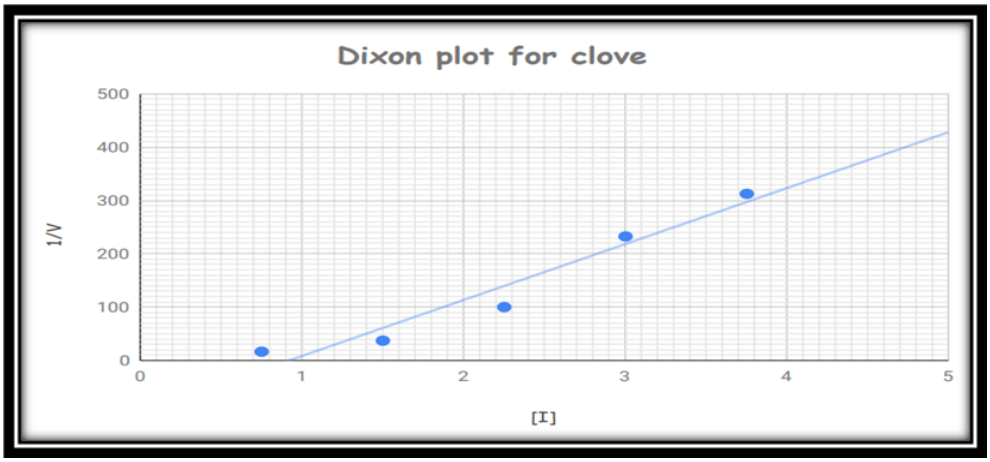

Figure 5: Dixon plot determining the inhibitory concentration of clove

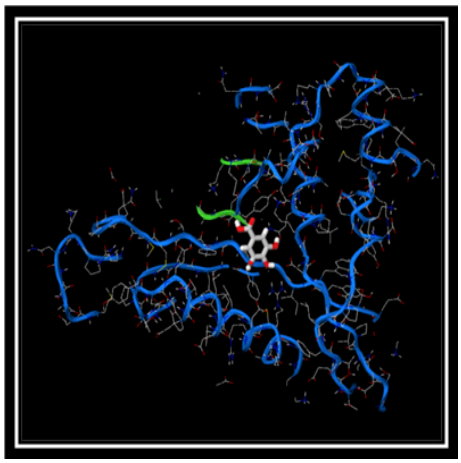

Figure 6: Gallic acid bound to HDC with binding affinity of -5.8

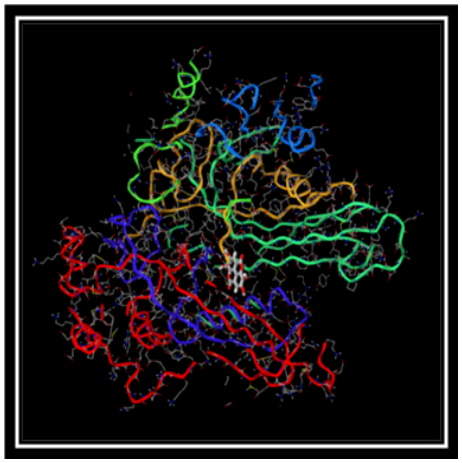

Figure 7: Ellagic acid bound to HDC with binding affinity of -7.9

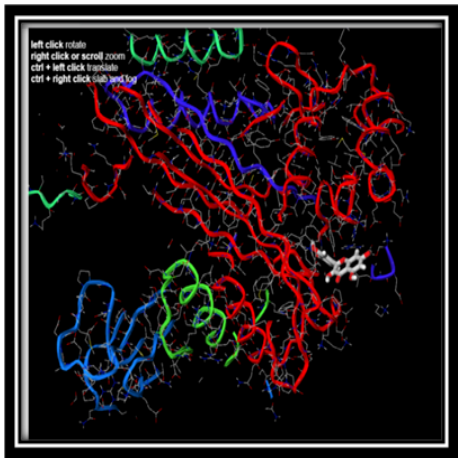

Figure 8: Quercetin bound to HDC with binding affinity of -7.2

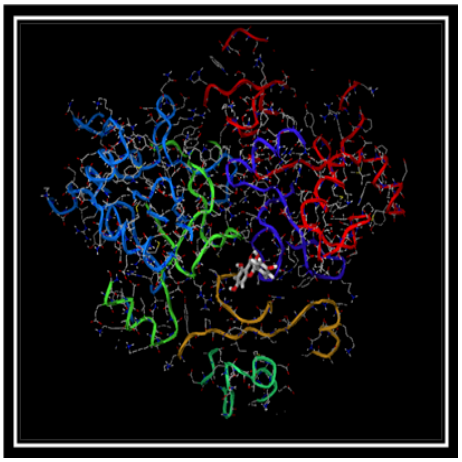

Figure 9: Kaempferol bound to HDC with binding affinity of -7.3

CONCLUSION

Currently, antihistamines are used to treat allergies. Antihistamines produce a range of side effects which can even be lethal. Antihistamines act by binding to the histamine receptors on the cells and preventing the binding of histamine, thus blocking the further process of a response. An alternate to antihistamines can be bought into existence by inhibiting the enzyme responsible for the production of histamine. The present study aims at inhibiting the enzyme histidine decarboxylase from the isolated flavonoids. The in-silico docking studies of the flavonoids with the enzyme HDC as the target was performed to identify the binding affinity of the ligand to their target and also analyse the residues involved in the binding of the ligand to the enzyme. Of the four flavanoids studied, Ellagic acid is identified as a very promising molecule as it showed a range of binding affinity from -6.0 to -7.9. It exhibits a strong binding with the target and can be a potential lead compound.

ACKNOWLEDGMENT

The authors would like to express their gratitude to the Department of Chemistry (PG Biochemistry) and the management of Mount Carmel College, Autonomous, Bengaluru for funding this project and offering their facilities for the analysis.

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