Curcumin and nano-curcumin as inhibitors for carbonic anhydrase purified from human erythrocyte

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Abstract

This study was aimed to inhibit carbonic anhydrase (CA) enzyme activity with curcumin (extract) and nano-curcumin (active compound) which had an antioxidant effect with low or no cytotoxic activity. Curcumin is natural and a bioactive compound found in the rhizome of turmeric (Curcuma longa L.), it can be used as a preventive and curative agent for many diseases such as diabetes, cancer, rheumatoid disease, allergy, and asthma and it has an effective role in eye diseases. Results of inhibition showed that nano-curcumin (active compound) was the most potent inhibitor than curcumin extract and the inhibitory percentage were 99 and 73.9 % respectively. Carbonic anhydrase enzyme purified from human erythrocytes by precipitation with 80% ammonium sulfate saturation, ion exchange chromatography and gel filtration chromatography using Sephadex G- 150 column. The specific activity of the crude enzyme was 9.1 U/mg protein reached 145 U/mg protein upon the last purification step. Characterization study of purified enzyme demonstrated that the optimum PH, PH stability, optimum temperature and temperature stability were 8, 8, 37 and (32-42 °C), respectively.

Keywords: *carbonic anhydrase, Inhibition, curcumin, nano-curcumin, human erythrocyte, purification.*

INTRODUCTION

Carbonic anhydrase (CA) is a zinc metalloenzyme that regulates the level of pH and CO2 in all living organisms. CA isozymes have a physiological function to facilitate the interconversion of CO2 and HCO3; so they play important role in different processes such as pH control and gas balance, calcification and photosynthesis. Also, it plays an important role in the transport of ions and regulation of pH in the eye, kidney, inner ear, and central nervous system [1]. CA can be found in animals, plants, algae, archaea, and eubacteria [2]. Many of human carbonic anhydrase isoforms are drug targets for the treatment of several disorders such as edema, glaucoma, epilepsy, obesity, and cancer, due to the presence of different isoforms in different tissues or organs that play diverse physiological functions, and their dysregulation or overexpression may lead to such pathological consequences because in the fact of carbonic anhydrase inhibitors have many applications in different pathologies [3]. Purification of enzyme is to isolate specific enzymes from other unwanted components. Purification of protein is crucial for characterization the structure and function of interest protein, purification and separation of enzyme depend on size of protein, binding affinity and physic-chemical properties [4].

Enzymes bind with different type of molecules, called enzyme inhibitors when molecule bind to enzyme and reduce the enzyme activity. While, it called enzyme activators when the molecule bind to enzyme and elevate the activity of enzyme. Carbonic anhydrase inhibitors used to treat people unable to tolerate eye drops and when the eye drop not effective in decrease the eye pressure [5]. Acetazolamide (AZM) used since the 1950s as carbonic anhydrase inhibitor for different medical conditions. For instance, it is highly efficient in treating glaucoma and treating or preventing acute mountain sickness [6]. But, in another study acetazolamide may cause several side effects such as: fatigue, nausea, vomiting, diarrhea, cramping and numbness [7]. natural product refer to any chemical substance that has been collected, isolated or extracted from living organisms and its widely used as medicine for the management variety of diseases which effect the human disease. So, curcumin is a natural substance obtained from turmeric and it has large amount of pharmacological and biological activities, development of new drugs from natural products were considered as a hard work, these require collection, extraction, isolation, purification and characterization of natural product with determination of pharmacological and toxicological effect [8]. Therefore, this study was aimed to inhibit CA enzyme activity with plant natural compound which had antioxidant effect with low or no cytotoxic activity and this aim was achieved by purification of carbonic anhydrase from erythrocyte of Iraqi glaucoma patients, Characterization of carbonic anhydrase enzyme, Methanolic extraction of curcumin plants (rhizomes and roots), Molecular docking for enzyme inhibitors (curcumin, ethyl iso-allocholate and xylopropamine), Assessment of the inhibitory effect of curcumin extract and nano-curcumin on the activity of carbonic anhydrase.

Methods:

Sample collection

A total of 120 blood samples were collected from Iraqi patients suffering from glaucoma from Ibn Alhaitham hospital in Baghdad during the periods from 1/12/2021 to 1/2/2022. Using a disposable syringe, 2-3ml of blood was collected by venipuncture in gel tubes. Samples were transferred under cooling conditions to the laboratory for analysis.

Preparation of erythrocyte membranes

Erythrocytes were isolated from fresh human blood, which was obtained from Ibn Alhaitham hospital in Baghdad following low-speed centrifugation at 2.000 rpm for 15 min to remove of plasma and buffy coat. The red cells were washed twice with NaCl (0.9%) and hemolyzed with 1.5 volumes of ice-cold water. Ghost and intact cells were then removed by high-speed centrifugation at 18.000 rpm for 30 min at 4°C, and the pH of the haemolysate adjusted to 8.7 with solid Tris [9].

Carbonic anhydrase activity assay

Carbonic anhydrase (CA) activity was assayed by adding 1.4 ml of potassium phosphate buffer, 1 ml of substrate (p-Nitrophenyl acetate), 0.5 ml of D.W and 0.1 ml of enzyme solution. Blank was prepared by the same method except the adding of enzyme solution [10]. The absorbance was measured at 405 by UV- visible spectrophotometer. CA activity was determined from the hydrolysis rate of p-nitrophenyl acetate to p-nitrophenol and calculated using the following eqution

$$\text{Activity of enzyme (U/ml)} \frac{Released\ nitrophenol\ in\ sample\ based\ on\ standard\ curve\ slope}{\text{reaction time} * \text{sample volume}}$$

One unit of CA activity was defined as the amount of enzyme that catalyzes the release of p- nitrophenol per minute from the substrate at room temperature 25° C. Protein concentration was determined by use standard curve of bovine serum albumin as standard [11].

Purification of carbonic anhydrase

Four steps were used for the purification of carbonic anhydrase from human erythrocytes: first by ammonium sulfate precipitation, Dialysis, Purification by ion exchange chromatography and gel filtration chromatography. The crude enzyme was subjected to 80% saturated ammonium sulfate precipitation. The precipitate was collected by centrifugation at 6000 rpm for 20 min. the precipitate was dissolved in suitable amounts of phosphate buffer then the precipitate was dialyzed in dialysis tube. The dialyzed fraction was applied to the diethylaminoethyl cellulose (DEAE-C) column. In the last step of purification by gel filtration chromatography, high activity fraction from ion exchange was collected and applied on Sephadex G-150.

Characterization of carbonic anhydrase:

Effect of PH on CA activity

The purified enzyme was added to substrate solution (p-Nitrophenyl acetate) prepared at different pH ranging from (5-9) including acetate buffer pH (5, 6), phosphate buffer pH (7, 8) and tris buffer pH (9) Enzyme activity was measured and the relationship between pH and enzyme activity was plotted.

Effect of pH on CA stability

The purified enzyme was pre-incubated in buffer of various PH (5-9) for 30 min at 37°C. After that the tubes were cooled in ice bath. Enzyme activity then measured and the remaining activity (%) for carbonic anhydrase was plotted against the pH value.

Effect of temperature on CA activity

Carbonic anhydrase activity was measured at different temperatures (27, 32, 37, 42 and 47 °C) and enzyme activity was plotted against the temperature.

Effect of temperature on CA stability

The purified enzyme was pre-incubated at different temperatures (27, 32, 37, 42 and 47 °C) for 30 min and immediately transferred into an ice bath. Carbonic anhydrase activity was determined and the remaining activity (%) was plotted against the temperature.

Molecular docking for enzyme inhibitors

The docking studies were carried out using Autodock tools (version 1.5.6) [12]. The active compounds were downloaded from PubChem database (Curcumin CID:969516, Ethyl Cholate CID:6452096 and Xylopropamine CID:26727). The cryptographic 3D structure of carbonic anhydrase was downloaded from RCSB protein data bank (PDB ID: 1BN1) the enzyme resolution for this model is 2.10 Å. Pre-docking treatment was performed for both of active compounds and receptor. For carbonic anhydrase enzyme, the co-crystalized ligand and water molecules were removed, the missing atoms were corrected and polar hydrogens were added. Energy minimization was conducted using Swiss protein viewer (version 4.1). Both of receptor and compounds were saved in pdbqt file extension. The grid box was parametrized with size dimensions (40* 40* 40) for x, y and z respectively with center values (-5.677, -0.512 and 18.341) Å. The Autodock vina parameters were set to default and 10 poses for each compound were generated. The poses were ranked based on scoring values and the pose with best scoring

value was selected. The docking results for generating 2D and 3D pictures were visualized using discovery studio (version 21.1.0.2) [13].

Inhibition of carbonic anhydrase

Carbonic anhydrase was inhibited by curcumin extract and Nanocurcumin according to Aditama with some modification as follow [14]:

Curcumin extract was dissolved by adding dimethyl sulfoxide (DMSO) and the volume was completed with distal water to 10 ml, while the same amount of acetazolamide (control) 250 mg dissolved in 10 ml distal water. After that, five concentrations (2, 4, 6, 8, 10 µg/ml) of curcumin extract, Nanocurcumin and acetazolamide were prepared. The assay mixture was prepared by adding 100 µl of each concentration of compounds (curcumin extract, Nanocurcumin and acetazolamide) to 100 µl of purified carbonic anhydrase enzyme. The mixture was incubated at room temperature for 15 min. after that, 0.1 ml of this mixture (sample) mixed with 1.4 ml of potassium phosphate buffer, 1 ml of substrate (p-Nitrophenyl acetate) and 0.5 ml of D.W were added, then the absorbance was measured at 405 nm by UV-visible spectrophotometer control was prepared using the same mixture in the absence of inhibitor. Carbonic anhydrase inhibitory activity was expressed as the inhibitory percentage calculated using the following equation:

Inhibitory percentage (%)=(control-sample)/control×100

Results and discussion:

The activity of crude carbonic anhydrase

The activity of crude carbonic anhydrase was determined in 50 ml of erythrocyte samples obtained from glaucoma patients. The result in table (1) shows that the specific activity of the crude enzyme was 9.1 U/mg Protein with 3.3 U/ml enzyme activity.

(Table 1) Purification steps for carbonic anhydrase purified from erythrocyte of glaucoma patients

Purification step	Volume (ml)	Enzyme activity (U/ml)	Protein concentration (mg/ml)	Specific activity (U/mg)	Total activity (U)	Purification (folds)	Yield (%)
Crude enzyme	50	3.3	0.36	9.1	165	1	100
Ammonium sulfate precipitation 80%	12	11.6	0.3	38.6	139.2	4.2	84.3
Ion exchange chromatography on DEAE-cellulose	18	6.6	0.12	55	118.8	6	72.7
Gel filtration chromatography on Sephadex- G150	18	5.8	0.04	145	104.4	16	63.2

Effect of PH on CA activity

The effect of PH on carbonic anhydrase activity was assayed at different values ranging from 5 to 9. The results showed that carbonic anhydrase activity increased by increasing the PH value until it reached pH=8, after that it began to decrease. So, the optimum PH of carbonic anhydrase activity was PH=8 and at this value, the specific activity of the enzyme was 6 U/ml as shown in figure (1), the activity of the enzyme gradually increase with increasing PH up to optimum then activity was decreased.

Most enzyme activities follow bell shape curve. Decrease to zero in region of strong alkaline and increase from zero in region of strong acid up to maximum value [15].

Effect of pH on CA stability

The purified carbonic anhydrase was incubated at 37°C for 30 min to determine the optimum PH that stable the enzyme. Results show the carbonic anhydrase was stable in a wide range of PH (5-9), but it was more stable at PH (7 and 8) because it had the highest remaining activity (100%) at PH 8 and this activity began to decrease to 90% at PH 9 as appeared in figure (2), so carbonic anhydrase can tolerate basic or alkaline conditions.

PH considered as determining factor in the expression of enzymatic activity so it alter the ionization state of the substrate or amino acid side chain. Most enzymes may undergo irreversible denaturation in high acidic or basic solution [16-18].

Effect of temperature on CA activity

Temperature can be considered as critical variable that determine the velocity of reaction, As shown in figure (3), carbonic anhydrase is active over range of temperatures from (27-47°C). However, the highest enzymatic activity was observed in 37°C since it was reached to 5.9 U/ml then the activity began decrease reached to 2.4 at 47°C.

For biological systems; the effects of temperature are complicated with contributions from protein stability and enzyme catalysis. Denaturation of enzyme, or lose characteristics of three- dimensional structure occur when temperature above or below the optimum temperature and that will drastically reduce the rate of reaction [19, 20].

Effect of temperature on CA stability

Carbonic anhydrase enzyme was incubated at different temperature (27-47°C) for 30 min, to determine the effect of temperature on stability of enzyme and the remaining activity was calculated after assaying enzyme activity. As appeared in figure (4) carbonic anhydrase was stable and maintain its activity (100%) at (32-42°C), then when the temperature was increased the remaining activity began to decrease until reached 40% at 52°C.

Molecular docking study for CA enzyme inhibitors

In silico docking studies were performed in order to underlying the possible inhibition mechanism of carbonic anhydrase by some of active compounds [21]. The docking results are list in table (2), based on binding affinities. The binding performance of active compounds was in order of curcumin > ethyl- iso allocholate > Xylopropamine. Curcumin possessed highest binding affinities due to forming multiple interactions with carbonic anhydrase's active site figure (5) including hydrogen bond between ARG246 and hydroxyl group. Another noticeable hydrogen bond was between LYS9 and carbonyl group. The highest affinity of curcumin could also be attributed to its

structural configuration which was orientated to fit at enzyme's pocket. The other two compounds [Xylopropamine (figure 6) and ethyl iso- allocholate (figure 7)] also show a moderate affinity toward carbonic anhydrase's pocket compared to curcumin. These figures show the docking profiles for the three active compounds at receptor active's site. The active compounds could act as antiglaucoma agents by reducing the activity of carbonic anhydrase [2].

It is useful method use to predict the interaction between ligand and receptor or to predict how enzyme interaction with small molecule (ligand), in this study I used curcumin and its active compounds (ethyl- iso allocholate and xylopropamine) against carbonic anhydrase and the result show curcumin has highest interaction with carbonic anhydrase while, Xylopropamine has lowest interaction with carbonic anhydrase enzyme.

Table (2): Binding energy for active compounds with carbonic anhydrase (PDB: 1BN1)

Compound Name	Binding Score (kcal/mol)	Interacting Residues
Curcumin	-7.4	A:TYR7, A:TRP:245, A:ASP243, A:ARG246, A:PRO13, A:GLU14, A:PRO247, A:HIS17, A:LYS9
Ethyl-iso alloCholate	-6.2	A:GLY132, A:GLN92, A:LEU198, A:HIS94, A:ASN67
Xylopropamine	-5.5	A:VAL121, A:LEU198, PHE131, A:ASN67, A:HIS94, A:HIS96, A:ALA65

Inhibition of carbonic anhydrase

Carbonic anhydrase enzyme after purification was subjected to inhibition by curcumin extracted from Curcuma longa L. and nano-curcumin active compound. Result in table (3) showed that inhibitory activity on carbonic anhydrase observed at all concentrations but in different percentage. Inhibition of carbonic anhydrase by curcumin extract increased with increasing concentration started from 10% at 2 µg/ml reached to 73.9% at 10 µg/ml. where as nano-curcumin (active compound) appeared as most potent inhibitor and gave 92% at 2 µg/ml reached to 99% at 10 µg/ml in a comparision to acetazolamide which is used as positive control exhibited carbonic anhydrase inhibitory activity 100 %.

(Table 3): Carbonic anhydrase inhibitory percentage by Curcumin extract and Nanocurcumin active compound compared to Acetazolamide as Control

Inhibitor	Carbonic anhydrase inhibition (%)				
	2 (µg/ml)	4 (µg/ml)	6 (µg/ml)	8 (µg/ml)	10 (µg/ml)
Acetazolamide/ Control	97	99	100	100	100
Curcumin extract	10	24.5	55.3	60.2	73.9

Nanocurcumin active compound	92	93.5	96	99	99

Figure (1): Effect of pH on activity of carbonic anhydrase purified from erythrocytes of glaucoma patients.

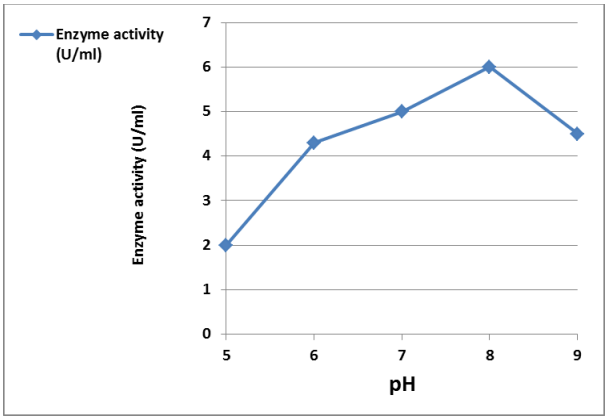


Figure (2): Effect of pH on stability of carbonic anhydrase purified from erythrocyte of glaucoma patients

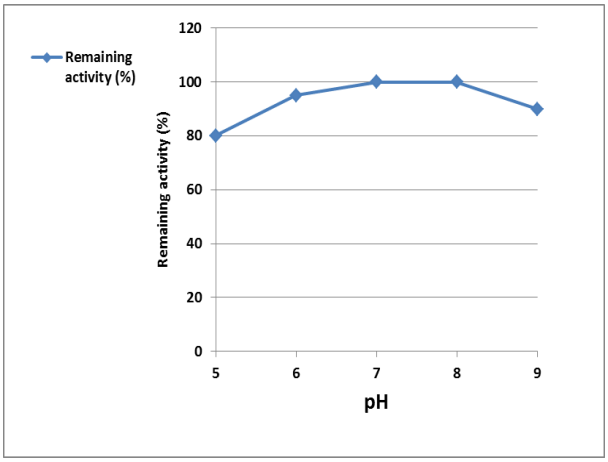


Figure (3): Effect of temperature on activity of carbonic anhydrase purified from erythrocyte of glaucoma patients

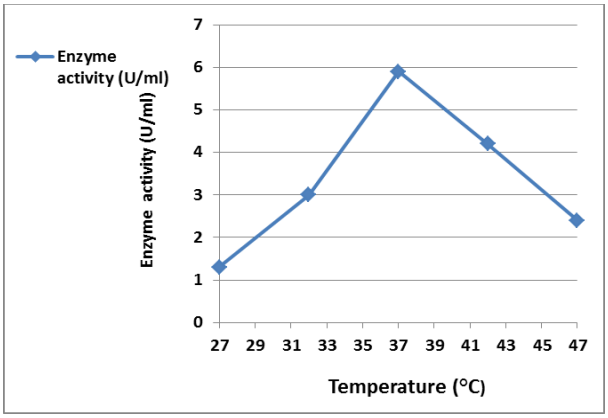


Figure (4): Effect of temperature on stability of carbonic anhydrase purified from erythrocyte of glaucoma patients.

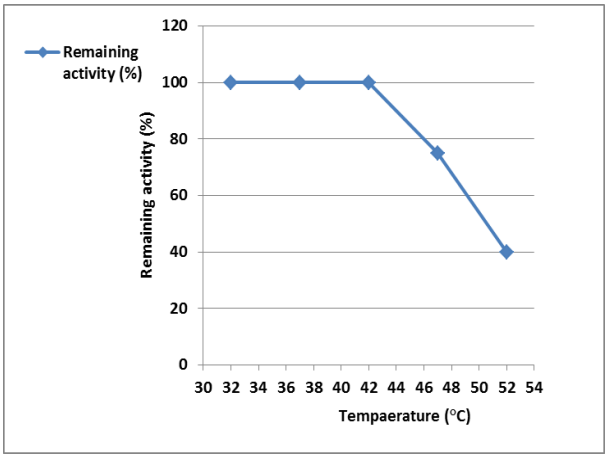


Figure (5): Docking profile of carbonic anhydrase enzyme (PDB ID: 1BN1) with curcumin (a): 3D structure of doct complex (b): scimatic 2D interaction of legand / receptor interaction.

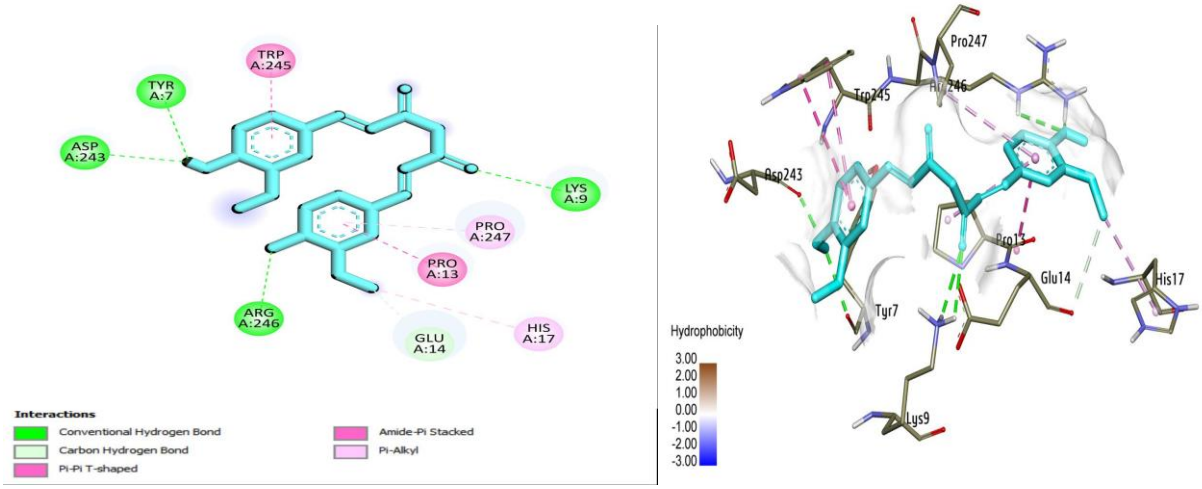


Figure (6): Docking profile of carbonic anhydrase enzyme (PDB ID: 1BN1) with xylopropamine (a): 3D structure of doct complex (b): scimatic 2D interaction of legand / receptor interaction.

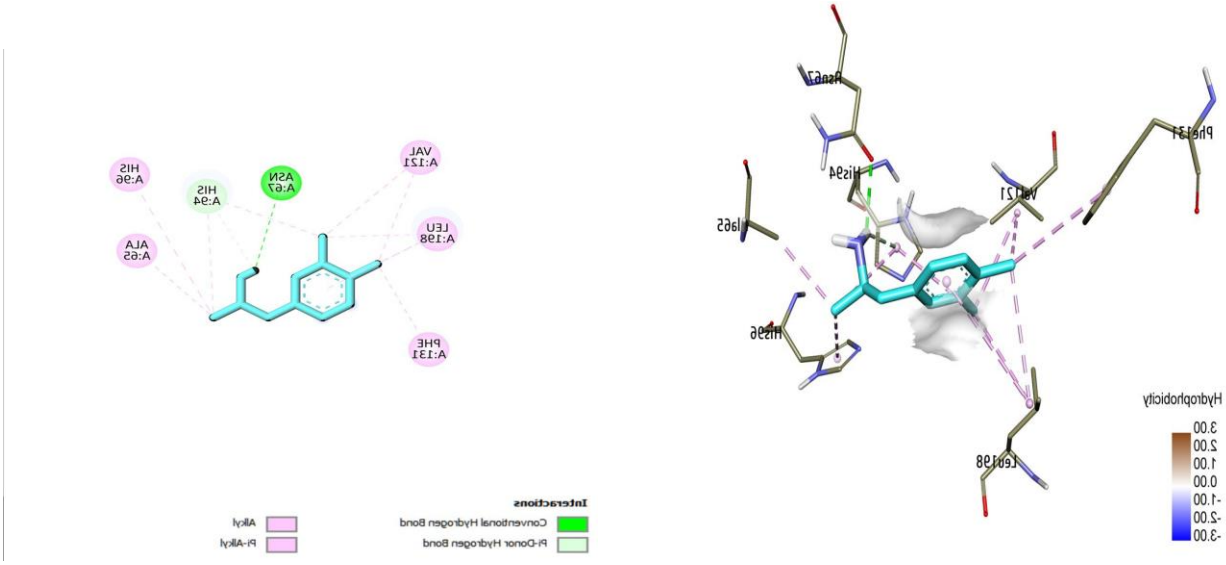
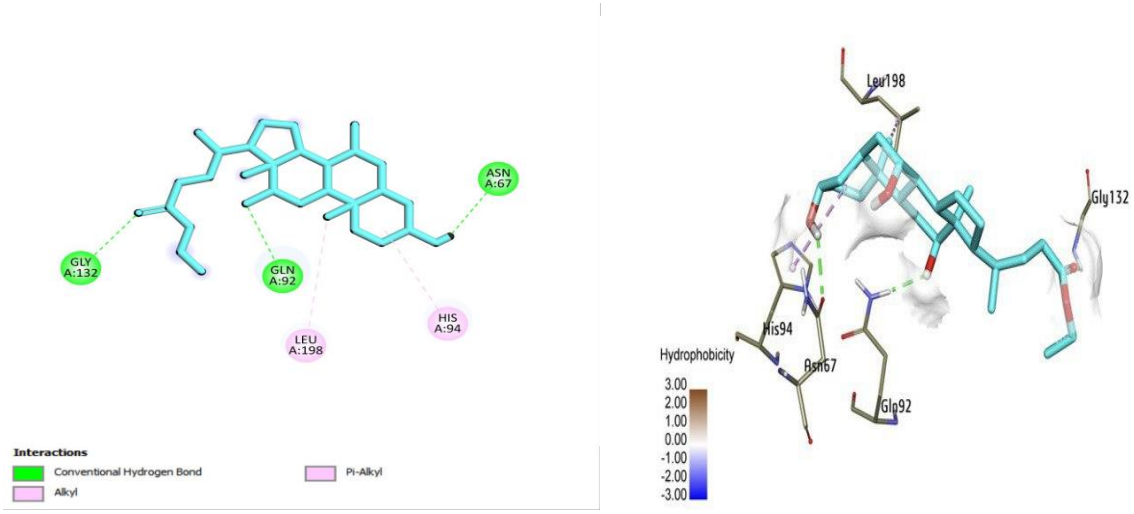


Figure (7): Docking profile of carbonic anhydrase enzyme (PDB ID: 1BN1) with ethyl iso-allocholate (a): 3D structure of doct complex (b): scimatic 2D interaction of legand / receptor interaction.



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