International Advance Journal of Engineering, Science and Management (IAJESM)
ISSN -2393-8048, July-December 2021, Submitted in July 2021, iajesm2014@gmail.com

Inhibitory effect of heavy metal ions on neem leaves CA

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Abstract

Carbonic anhydrase is aubiquitous metalloenzyme that catalyzes the reversible hydration of CO_2 to HCO_3^- and H^+. Metals are essential to this metalloenzyme's bioactivity, however their interactions with CA are yet not entirely understood. In this work CA from neem leaves has been extracted and inhibitory effect of different heavy metals (Cd^{2+}, Hg^{2+}, Pb^{2+}, Sr^{2+},and Ce^{3+}) on this enzyme is being studied. The results indicated that even very low concentrations of these heavy metals showed inhibitory effects on neem leaves CA. The enzyme is shown to be most inhibited by Hg^{2+}, least inhibited by Pb^{2+} and Ce^{3+}, and moderately inhibited by Sr^{2+} and Cd^{2+} among the heavy metals investigated. From Michaelis-Menten and Lineweaver-Burk curves, the type of inhibition by these metal ions were investigated. It has been discovered that Sr^{2+} is an uncompetitive divalent metal ion while Cd^{2+}, Hg^{2+}, and Pb^{2+} are competitive in nature and trivalent metal ions like Ce^{3+} is non-competitive in nature.

1. Introduction

Carbonic anhydrase(CA) is azinc-containing enzyme that promotes the rapid inter-conversion of carbon dioxide (CO_2) and water (H_2O) into carbonic acid (H_2CO_3), protons (H^+) and bicarbonate ions (HCO_3^-) or catalyzes the reversible hydration of carbon dioxide. The reaction can be summarized below:

$$CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$$

Cow red blood cells were the first to exhibit carbonic anhydrase, which was discovered in 1933. All tissues of mammals as well as plants, algae, and bacteria have been found to have large amounts of carbonic anhydrase. In all kingdoms of life, carbonic anhydrase is present and plays a role in respiration and photosynthesis in eukaryotes as well as cyanate degradation in prokaryotes (Hassan et al., 2013).The reaction catalyzed by CA plays a role in a variety of physiological and pathological processes, such as bone resorption, calcification, tumorigenicity, pH and CO_2 homeostasis, respiration, transport of CO_2 and bicarbonate between metabolizing tissues and lungs, and electrolyte secretion in various tissues and organs (Supuran, 2008).

All living things are eventually exposed to heavy metals from numerous sources. In fact, it is known that heavy metals can interfere with the catalytic activities of enzymes including CA by reducing their activity(Kocyigit et al., 2018)(Demirdag et al., 2012)(Kirici et al., 2016)(Ceyhun et al., 2011). Heavy metals, even in small concentrations, is deadly or toxic for e.g., Mercury (Hg), cadmium (Cd), arsenic (As), chromium (Cr), thallium (Tl), strontium (Sr), and lead (Pb).Massive increase in human exposure to heavy metals has been brought about by the industrial activities of the previous century. The most frequent heavy metals to cause human poisoninghave been mercury, lead, chromium, cadmium, and arsenic. Poisonings that are either acute or chronic may accompany exposure to water, air, or food. These heavy metals bioaccumulate in the body and have a variety of harmful effects on various human tissues and organs. Apoptosis, differentiation, growth, proliferation, and other biological functions are all affected by heavy metals. Genomic instability is a result of some hazardous metals, including chromium, cadmium, and arsenic(Balali-Mood et al., 2021).

The IC_{50} values for Co^{2+}, Cu^{2+}, and Fe^{2+} in sheep erythrocytes CA were 0.42, 0.63, and 1.22 mM, respectively (Kocyigit et al., 2018). Additionally, IC_{50} values of 0.66, 0.75, 0.00041, 1.44, and 0.058 mM for Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+}, and Zn^{2+}, respectively, were discovered in sheep liver CA(Demirdag et al., 2012). IC_{50} values for Cd^{2+}, Fe^{3+}, and Ni^{2+} in fish gills were also discovered to be 0.19, 0.14, and 0.92 mM, respectively(Kirici et al., 2016). For the respective functions of Al^{3+}, Co^{2+}, Cu^{2+}, and Zn^{2+} in fish liver, IC_{50} values of 0.07, 0.32, 0.07, and 0.39 mM were discovered(Ceyhun et al., 2011).In camel liver CA I_{50} values of 8.19, 10.23, 0.18, 2.48 and 0.59 was found for Cd^{2+}, Co^{2+}, Cu^{2+}, Ni^{2+} and Zn^{2+}(Chafik et al., 2020). In plant CA, metal inhibition study have been reported in pea leaves from Hg^{2+}, Pb^{2+}, Mn^{2+}, Mg^{2+}, Zn^{2+},

Fe^{2+} and Ni^{2+}, and in tea leaves CA from Hg_2^{2+}, Sb^{3+} and Al^{3+}(Kisiel and Graf, 1972)(Demir et al., 1997).
This study aims at study of inhibition of neem leaves CA by heavy metals and determine their nature of inhibition

2. Materials and methods

2.1. Chemicals required

p-nitrophenyl acetate, Tris-HCl buffer,$CdCl_2$,$HgCl_2$,$Ce(NO_3)_3$,$Sr(NO_3)_2$,$Pb(NO_3)_2$ were purchased from SRL.

2.2. Preparation of enzyme extract

The *Azadirachta indica* leaf was grinded by mortar and pestle.The juice was extracted and was then stored in refrigerator for further use.

2.3. Enzyme assay

The activity of carbonic anhydrase was determined spectrophotometrically (SHIMADZU UV 2600) by using p-nitrophenyl acetate as the substrate. The formation of p-nitrophenol was monitored at λ = 348 nm.Neem as an enzyme source: The reaction solution of 1 ml consisted of 64mM Tris-HClbuffer pH=8, 1.02mM p-nitrophenyl acetate as the substrate, 20µL enzyme solution as per standard protocol(Demir et al., 1997).

2.4. Inhibition studies

The steady-state velocity of the enzyme-catalyzed reaction was measured at different concentrations of various heavy metal ions like divalent(Cd^{2+}, Hg^{2+}, Pb^{2+}, Sr^{2+})and trivalent(Ce^{3+}) in the range of 2mM to 10mM at a fixed enzyme concentration. Graphs were plotted to determine the nature of the enzyme inhibition.
The Lineweaver-Burk plot was widely used to determine important terms in enzyme kinetics, such as Km and Vmax, before the wide availability of powerful computers and non-linear regression software. The y-intercept of such a graph is equivalent to the inverse of V_{max}; the x-intercept of the graph represents -1/Km as shown in **fig. 1**.

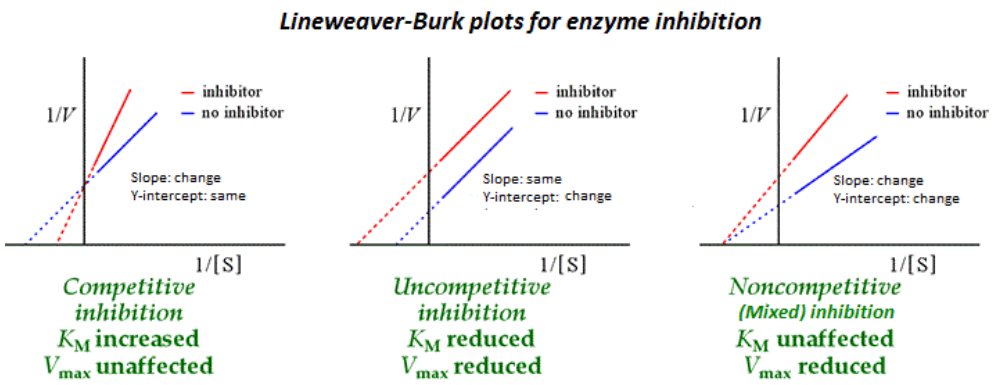

Fig 1. The Lineweaver-Burk plot for enzyme inhibition

3. RESULT AND DISCUSSION

3.1. Enzyme assay

Carbonic anhydrase enzyme assay was performed using p-nitrophenyl acetate as the substrate monitored atλ = 348nm.The activity of the neem CA leaves was found to be 2.88U/ml.

3.2. Inhibition of carbonic anhydrase by differentheavy metals

Inhibitors are a very helpful tool for understanding how enzymes work. Information regarding the binding characteristics of the enzyme can be obtained by examining which substances inhibit an enzyme and which do not. Additionally, inhibitors can reveal details regarding substrate selectivity and the kinds of processes the enzyme is capable of catalyzing. Understanding each enzyme in the system and how each reaction fits with the others is necessary for understanding the metabolic pathway, which is the second important area of study for inhibitors. Using inhibitors as probes to determine each enzyme's function is one

approach to doing this. As a result, one of the key mechanisms for physiological enzyme regulation is inhibition(Spector and Hajian, 1981)(Basumatary et al., 2020).
CA enzyme was inhibited in the presence of different heavy metal ions as shown in the **fig2**. It was seen that the Carbonic anhydrase enzyme was highly inhibited by Hg^{2+} and less inhibited by Sr^{3+} among the metal ions investigated.

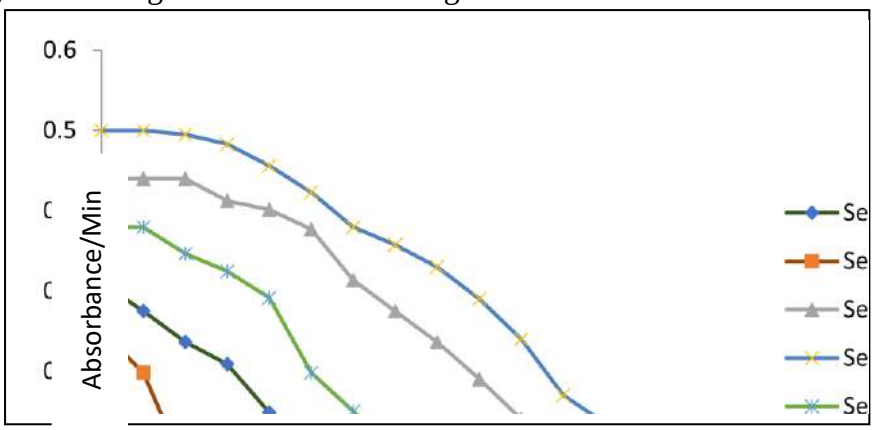

Fig2. ɔnic anhydrase catalysed reaction in the presence of different heavy metal ions. Series 1- Cd^{2+}, Series 2- Hg^{2+}, Series 3- Ce^{3+}, Series 4- Pb^{2+}, Series 5-Sr^{2+}

3.3.Nature of inhibition by different heavy metals

3.3.1. Michaelis-Menten and Lineweaver burk plot ofCA catalyzed reaction in the presence of $CdCl_2$.

[Metal ion]

Nature of inhibition was studied by plotting Michaelis–Menten curve and double reciprocal plots were made in the presence of different concentration of Cd^{2+}. K_m values for 0mM, 2mM and 4mM are 0.40, 0.95 and 1.23, respectively.

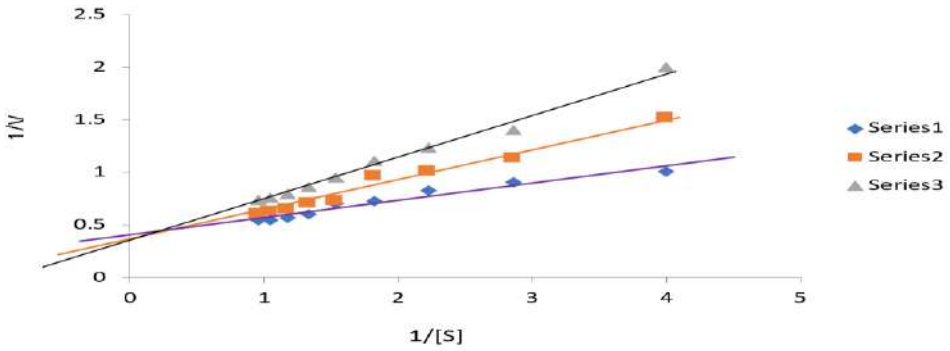

Fig 3. The figure indicates that the nature of inhibition of Cd ion is competitive. Series 1- enzyme, Series 2- $CdCl_2$ (2mM), $CdCl_2$ (4mM)

The activity of enzyme carbonic anhydrase is inhibited by the presence of metal ion Cd^{2+} as shown in **fig 3**. It is clear from **fig 3** that the nature of inhibition by Cd^{2+} ion is competitive type i.e., the inhibitor bind to the free enzyme but not to the enzyme-substrate complex.The competitive inhibition is schematically shown below.In this kind of inhibition, the enzyme cannot be bound by both the substrate and the inhibitor simultaneously. Both the substrate and the inhibitor have trouble getting to the enzyme's active site because the inhibitor binds to the enzyme's active site and prevents the substrate from binding. By increasing the likelihood of the enzyme and substrate interacting, more substrate can be added to the reaction to solve this issue. This then changes the K_m value of the enzyme while keeping the V_{max} value of the enzyme constant. When the inhibitor binds to the enzyme, the slope of the line will be altered because K_m will vary(Basumatary et al., 2020).

3.3.2.Michaelis Menten and Lineweaver burk plot ofCA catalyzed reaction in presence of $HgCl_2$

The Michaelis-Menten curve of carbonic anhydrase catalyzed reaction and Lineweaver-Burk plot was studied in the presence of different concentration of Hg^{2+} ions.
K_m values for 0mM, 2mM, 4mM are 0.39, 3.83 and 59.46, respectively.

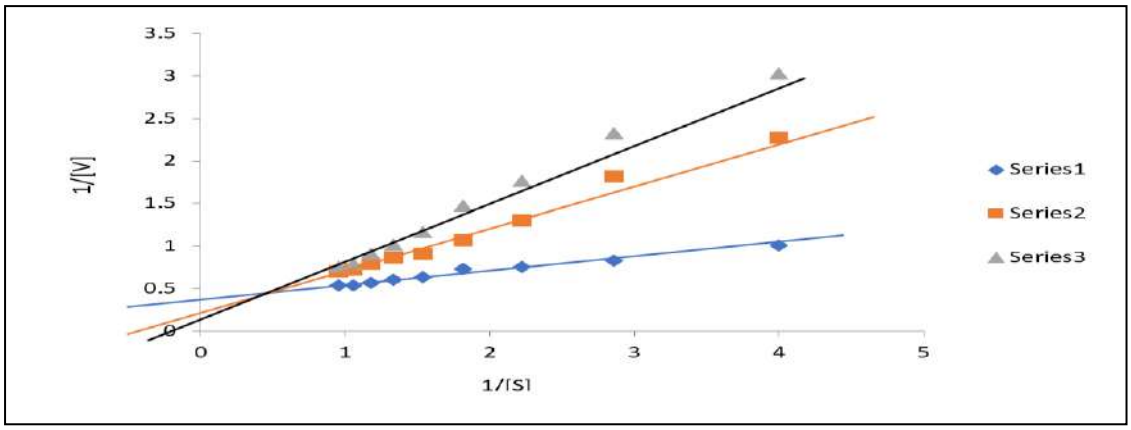

Fig 4. Lineweaver-burk plot of carbonic anhydrase catalysed reaction inhibited by Hg^{2+} ion. Series 1-Enzyme(mM), Series 2- $HgCl_2$(2mM), Series 3- $HgCl_2$(4mM)

The activity of enzyme carbonic anhydrase is inhibited by the presence of metal ion Hg^{2+} as shown in **fig 4**.It is clear from **fig 4** that the nature of inhibition by Hg^{2+} ion is competitive type i.e., the inhibitor bind to the free enzyme but not to the enzyme-substrate complex.
In this kind of inhibition, the enzyme cannot be bound by both the substrate and the inhibitor simultaneously. The inhibitor blocks the substrate from binding to the enzyme's active site, making it harder for the substrate to reach the enzyme's active site. By increasing the likelihood of the enzyme and substrate interacting, additional substrate may be added to the process to solve this issue. This changes the enzyme's K_m value while maintaining its V_{max} value. When the inhibitor binds to the enzyme, the value of Km will vary, which will have an impact on the slope of the line(Basumatary et al., 2020).

3.3.3. Michaelis-Menten curve and Lineweaver burk plot of CA catalyzed reaction in presence of $Ce(NO_3)_2$

The Michaelis-Menten curve of carbonic anhydrase catalysed reaction and Lineweaver-Burk plot was studied in the presence of different concentration of Ce^{2+} ions.
K_m values for 0m M, 2m M, 4m M are 0.55, 0.38 and 3.05 respectively.

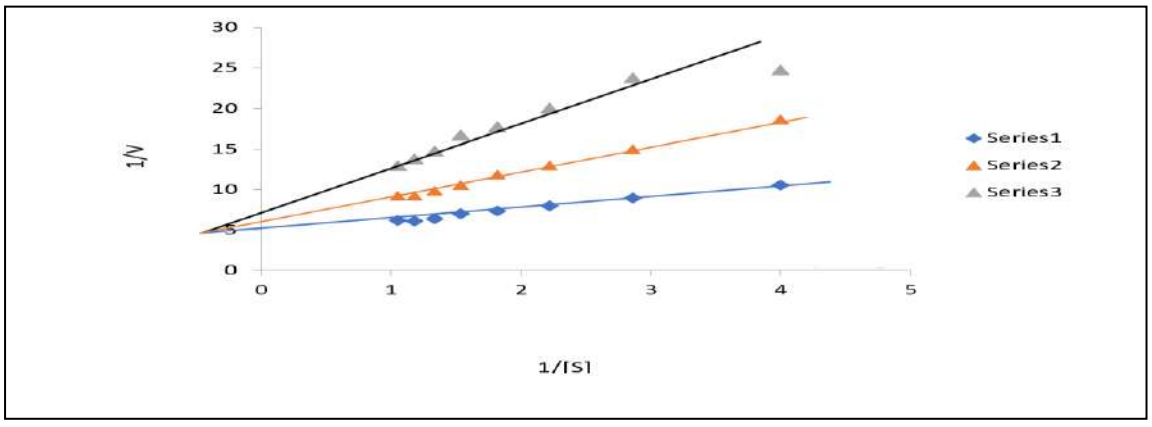

Fig5.Lineweaver-Burk plot of carbonic anhydrase reaction inhibited by Ce^{3+}. Series 1-enzyme, Series 2- $Ce(NO_3)_2$ (2mM), $Ce(NO_3)_2$(4mM)

The activity of enzyme carbonic anhydrase is inhibited by the presence of metal ion is evident from **fig 5**. It is clear from **fig 5** that the nature of inhibition by Ce^{3+} ion is Non competitive type i.e. the inhibitor bind to the enzyme at location other than the active site. In the other cases, it is thought that the inhibitor's binding alters the structure of the enzyme molecule, distorting the active site and blocking substrate reaction. They are also known as allosteric inhibitors

since the allosteric site refers to the location where the inhibitor binds to the enzyme(Spector and Hajian, 1981).

3.3.4. Michaelis-Menten curve and Lineweaver burk plot of CA catalyzed reactionin presence of $Pb(NO_3)_2$

Nature of inhibition was studied by plotting Michaelis–Menten curve and double reciprocal plots were made in the presence of different concentrations of Pb^{2+} ions.
K_m values for 0mM, 2m M, 4mM are 0.38, 0.50 and 1.22, respectively.

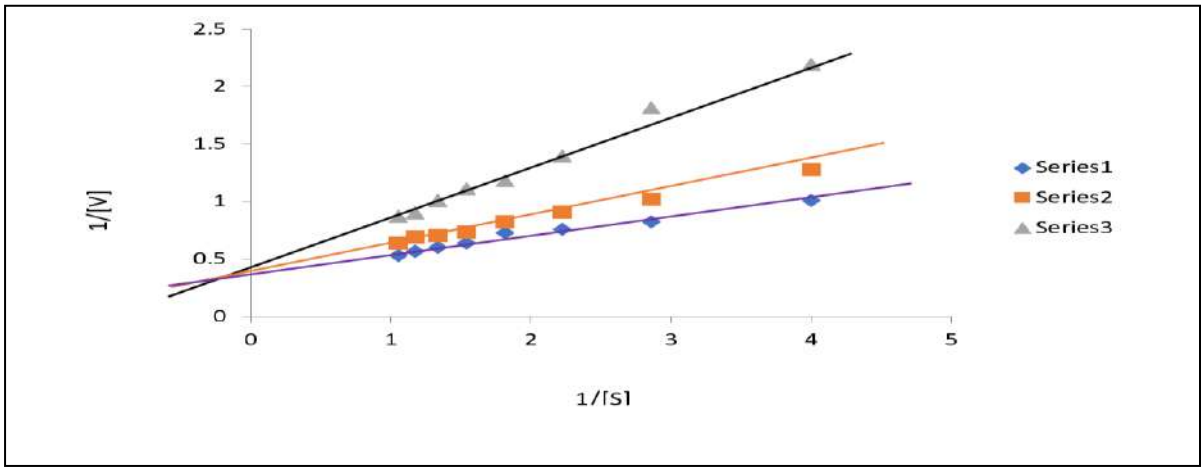

Fig 6.Lineweaver-burk plot of carbonic anhydrase catalysed reaction inhibited by Pb^{2+}. Series 1- enzyme, Series 2- $Pb(NO_3)_2$ (2mM), $Pb(NO_3)_2$ (4mM)

The activity of enzyme carbonic anhydrase is inhibited by the presence of metal ion Pb^{2+} as shown in **fig 6**.It is clear from **fig 6**that the nature of inhibition by Pb^{2+} ion is competitive type i.e. the inhibitor bind to the free enzyme but not to the enzyme-substrate complex. The competitive inhibition is schematically shown below.
In this kind of inhibition, the enzyme cannot be bound by both the substrate and the inhibitor simultaneously. Both the substrate and the inhibitor have trouble getting to the enzyme's active site because the inhibitor binds to the enzyme's active site and prevents the substrate from binding. By increasing the likelihood of the enzyme and substrate interacting, additional substrate may be added to the process to solve this issue.
This changes the enzyme's Km value while maintaining its Vmax value. When the inhibitor binds to the enzyme, the value of Km will vary, which will have an impact on the slope of the line(Basumatary et al., 2020).

3.3.5. Michaelis-Menten curve and Lineweaver burk plot of CA catalyzed reaction in presence of $Sr(NO_3)_2$

Nature of inhibition was studied by plotting Michaelis–menten curve and double reciprocal plots were made in the presence of different concentration of Sr^{2+} ions.
Km values for 0m M, 2m M, 4m M are 0.38, 0.52 and 0.39 respectively.

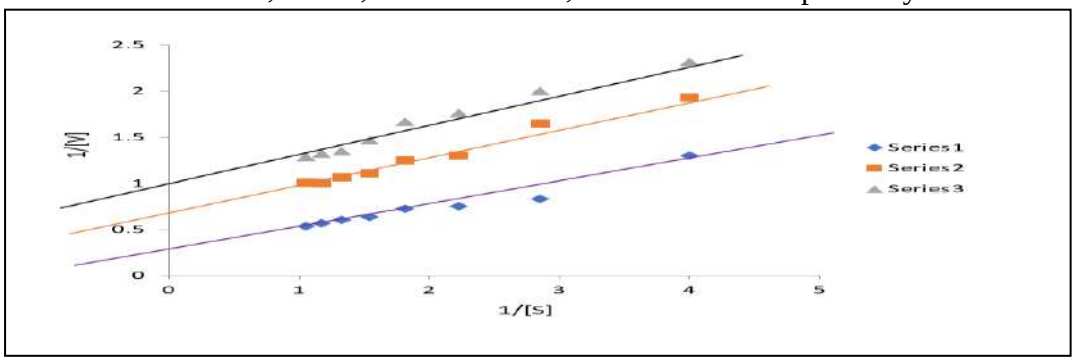

Fig 7. Lineweaver-burk plot of carbonic anhydrase catalysed reaction inhibited by Sr^{2+}. Series 1- enzyme, Series 2- $Sr(NO_3)_2$ (2mM), $Sr(NO_3)_2$ (4mM)

Carbonic anhydrase activity is inhibited in the presence of Sr^{2+} as shown in **fig7**.It is evident from **fig 7** that Sr^{2+} is an uncompetitive inhibitor and bind only to enzyme-substrate complex [ES].Uncompetitive or anticompetitive inhibition refers to an enzyme inhibitor that only interacts with the ES complex, the complex formed between the substrate and catalyst. In reactions involving at least two substrates or products, uncompetitive inhibition typically occurs. The downstream catalytic species produced by the uncompetitive inhibitor simply perceive and join with the ES complex, distancing itself from the free enzyme. Thus, uncompetitive inhibition requires the formation of an ES complex to demonstrate enzyme binding, and inhibition of enzyme activity is characterized by a drop in the values of both substrate K_m and V_{max}(Basumatary et al., 2020).

7. CONCLUSION

Through the course of this work the nature of inhibition of carbonic anhydrase enzyme obtain from neem leaves were studied in the presence of different heavy metals which include divalent Cd^{2+}, Hg^{2+}, Pb^{2+}, $Sr^{2+}$and trivalent Ce^{3+}. It is found after plotting a graph between absorbance/min and metal ion that, Hg^{2+} inhibit the enzyme the most and Pb^{2+} and Ce^{3+} the least, while Cd^{2+} and Sr^{2+} moderately inhibit the enzyme among the heavy metals studied. The nature of inhibition by different metal ions were studied by a plot of Michaelis-Menten and Lineweaver-Burk curves.It is found that divalent metal ion like Cd^{2+}, Hg^{2+}, Pb^{2+} are competitive in nature and Sr^{2+} is uncompetitive in nature. And trivalent metal ion like Ce^{3+}shows non-competitive inhibition.

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