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Synthesis, Characterization of New Azo Compounds and Studies Effect on the Ach Enzyme (*Invitro*)

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Abstract

Three azo compounds were synthesized in two different methods, and characterized by FT-IR, HNMR and (UV-Vis) spectra, melting points were determined. The inhibitory effects of prepared compounds on the activity of human serum cholinesterase have been studied in vitro. Different concentrations of these compounds were used to study the type of inhibition. The results form line weaver-Burk plot indicated that the inhibitor type was noncompetitive with a range (33.12-78.99%).

Keywords: azo compound, inhibitor, cholinesterase, invitro.

Introduction

Synthetic azo compounds were widely used in different application fields, such as medicines, cosmetics, food, paints, plastics, shipbuilding, automobile industry, cable manufacture, etc[(1)]. However the traditional application field of the synthesis azo dyes still remains the textile industry, and the finishing of fibrous material in order to impart simultaneously with coloriation[(2)].The biological importance of azo compounds is well known their use as inflammatory, antifungal, and anticancer[(3)].In this research use two methods to confuse azo compounds, where be attend a composite compound 4,4-azo di benzoic acid shorthand way group nitro in para nitro benzoic acid with glucose and sodium hydroxide[(4)], and attended the two compounds 4-(4-nitrobenzene azo)-3-aminobenzoic acid[(5)], and compound 5-(2-benzioc acid azo)-8-hydroxyquinolone[(6)], in way that duplication. Hundreds of compounds have been synthesized and tested as a cholinesterase inhibitors, they belong to different type of organic and organo phosphorous[(7)].

Acetyl cholinesterase (EC.3.1.1.7), AChE, also known as RBC cholinesterase, erythrocyte cholinesterase or (most formally) specific cholinesterase[(8,9)]. AChE, is an enzyme which is critical to the function of animals form ants to elephants. This enzymes sole responsibility is to break down the neurotransmitter acetylcholine. Acetylcholine sends message between nerves, signaling muscle concentration. If the neurotransmitter was not broken down after it had served its function, the muscle involved would be able to relax and it's could other problems[(10)]. The enzyme acetyl cholinesterase can be found in the synaptic cleft, the gap between nerve cells through which information flows. When acetylcholine passes through the enzyme breaks in down in to choline and acetic acid[(11)], after it has served its function, ensuring that the neurotransmitter dose not continue to float through the body. The choline and acetic acid are recycled by the body to make more acetylcholine so that reserves of neurotransmitter will be ready when the body needs it, acetyl cholinesterase can break down acetylcholine in microseconds, working rapidly to keep the synaptic cleft clear so that mixed massages do not occur [(12)].

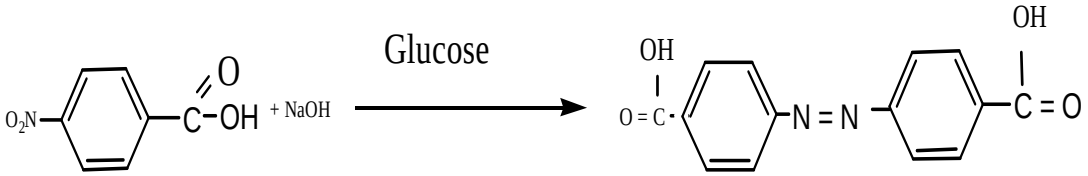
Experimental

1. Instrumentation

UV- Vis spectra were recorded on a (Shimadzu UV- 160A) Ultra Violet-Visible Spectrophotometer. IR-spectra were taken on a (Shimadzu, FT-IR-8400S) Fourier Transform Infrared Spectrophotometer (4000-400) cm^{-1} with samples prepared as KBr discs. Atomic absorption was obtained using a (Shimadzu A.A-160A) Atomic Absorption/Flame Emission Spectrophotometer. The ^{1}HNMR spectra were obtained on a (Brucker-300 MHz Ultra Shield) University of Al- al- Bayt using DMSO as a solvent and (TMS) as a reference. Microelemental analysis (C.H.N) was performed in Al- al- Bayt University- Jordan using (Euro vector EA 3000A Elemental Analyser).

2. Synthesis of 4,4-azo dibenzoic acid [4]

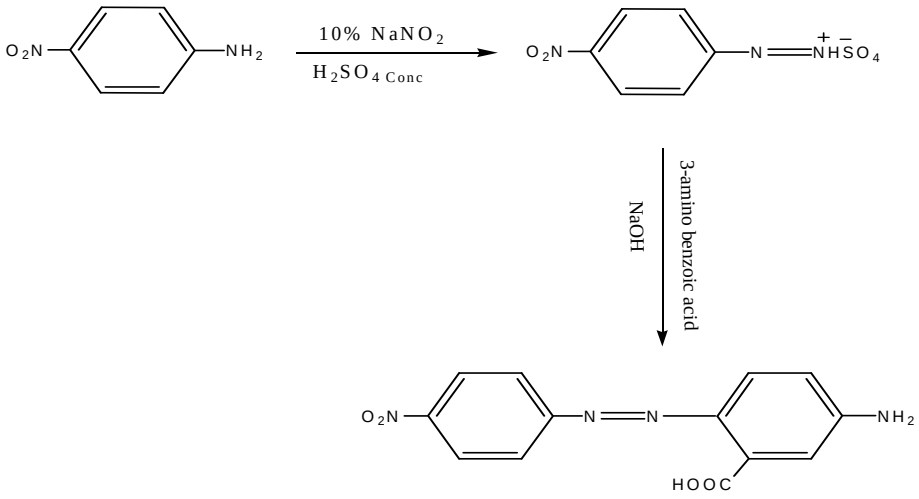
Synthesis of 4,4-azodibenzoic acid was obtained by condensation of P-Nitro benzoic acid with itself under air oxidation. The reaction is shown in scheme (1).



Scheme (1):-Preparation of the 4,4-azo dibenzoic acid.

3.Synthesis of 4-(4-Nitrobenzene azo)-3-amino benzoic acid[5]

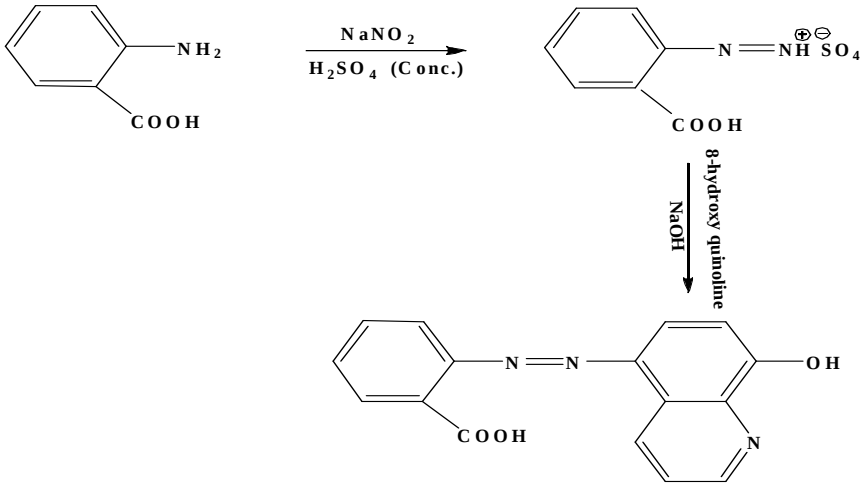
(0.342g,1mmole) of 4- nitro aniline was dissolved in a mixture of (2ml) sulphuric acid, (10ml) ethanol and (10ml) distilled water, and diazotized at 5°C with sodium nitrite solution. The diazo solution was added drop wise with stirring to a cooled ethanolic solution of (0.345g,1mmole) of 3-aminobenzoic acid. (25ml) of (1M) sodium hydroxide solution was added to the dark colored mixture. The precipitate was filtered off and washed several times with (1:1) ethanol: water, mixture then left to dry. The reaction is shown in scheme (2).



Scheme (2):-Preparation of the 4-(4-Nitrobenzene azo)-3-amino benzoic acid.

4. Synthesis of 5-(2-Benzioc acid azo)-8-hydroxyquinoline[6]

The ligand was synthesis according to the general method by dissolving (0.34g ,1 mmole) of 2-amino benzoic acid in a mixture consisting of (2ml) of sulphuric acid , (10ml) ethanol and (10ml) of doubly distilled water. The mixture was cooled to 5 °c (10ml) of 10% sodium nitrite was added dropwise with stirring in order to obtain the diazonium salt solution .After 30 min. the diazonium solution was slowly added to a cooled solution of (0.36g , 1mmole) of 8-hydroxy quinoline to obtain the ligand. The dark colored mixture was neutralized by sodium hydroxide and the solid precipitate was filtered off and washed several time with (1:1) ethanol : water, mixture then left to dry .The reaction is shown is scheme(3).



Scheme (3):-reparation of the 5-(2-Benzioc acid azo)-8-hydroxyquinoline.

5. Enzyme assay

This compounds were dissolved in dimethyl sulphoxide (DMSO) and stock solutions were made for each compound-different volumes from these stock were added to the assay mixture and the enzyme activity was determined according to a slightly modified WHO procedure[13].Volume of 2-250ml of phosphate buffer ($_p$H=7.3, 0.2M), 50 ML OF DTNB solution and 10 ml serum was served as the assay mixture. In a 3 ml curette, 2 ml of this mixture was taken and 34 ml of substrate (acetylthio cholin iodide) was added and the absorbance was measured at 430 nm. DMSO was used as avehicle solution (control) and showed no inhibitory effect on the activity of the enzyme [14].

Result and Discussion

1. Spectroscopic studies

1.1. 4,4-azo dibenzoic acid

^{1}HNMR spectrum of the 4,4-azodibenzoic acid in DMSO shows multiple signals at (δ=7.6-8.2 ppm) refers to aromatic protons .On the other hand, the signal at (δ=12.0 ppm) due to proton of carboxylic group[4].

1.2. 4-(4-Nirto benzene azo)-3-amino benzoic acid

The ^{1}HNMR spectrum of the 4-(4-nitrobenzene azo)-3-aminobenzoic acid in DMSO shows multiplet signal at (δ=6.617-8.431 ppm) refers to aromatic protons. The signal at (δ=12.075 ppm) due to proton of carboxylic group. Whereas, the signal at (δ=3.887 ppm) is assigned to δ(NH_2) and the signal peak at(δ=2.50 ppm) referred to DMSO-d6[15] .

The IR spectrum of the 4-(4-nitrobenzene azo)-3-aminobenzoic acid exhibited broad band at 3379 cm^{-1} was assigned to the stretching vibration of υ(OH) group[2]. The bands at 3296 cm^{-1} and 3116 cm^{-1} due to the stretching mode of υ(NH_2)[16]. The strong bands at 1666 cm^{-1} and 1558 cm^{-1} due to υ_{as}(COO) asymmetric and υ_s(COO) vibration[17]. Band characteristic of the azo bridge[18] vibration at 489 cm^{-1}.

The UV-ViS spectrum of an ethanolic solution of the 4-(4-nitrobenzene azo)-3-aminobenzoic acid (10^{-3}M) display mainly two peaks, the first peak was observed at 265 nm was assigned to the moderate energy π-π* transition of the aromatic rings. Other peak (λ_{max}) was observed at the 405 nm was referred to the π-π* transition of intermolecular charge-transfer taken place from benzene through the azo group(-N=N)[19]

1.3:- ***5-(2-Benzoic acid azo)-8-hydroxy quinoline***

The ^{1}HNMR spectrum of the 5-(2-Benzoic acid azo)-8-hydroxy quinoline in DMSO shows multiplet signals at (δ=6.915-8.097 ppm) refers to aromatic protons .On the other hand, the signal at (δ=6.630 ppm) due to proton of

phenol. Whereas, the signal at (δ=12.432 ppm) is assigned to proton of carboxylic group and the signal peak at(δ=2.495ppm) referred to DMSO-d6[20] .
The IR spectrum of the 5-(2-Benzoic acid azo)-8-hydroxy quinoline exhibited broad band at 3414 cm^{-1} has been assigned to the stretching vibration of υ(OH) of carboxyl group[21]. The band at 3387 cm^{-1} referred to υ(OH) of phenol[22]. Strong band has been observed at 1620 cm^{-1} ascribed to the υ(C=O) for the carboxyl group[23]. The strong bands at 1589 cm^{-1} and 1496 cm^{-1} due to υ(COO) asymmetric and υ(COO) symmetric vibration[18] Bands characteristic[24] of the azo bridge vibration at 1454 cm^{-1} and 1411 cm^{-1}.

The UV-Vis spectrum of an ethanolic solution of the5-(2-Benzoic acid azo)-8-hydroxy quinoline (10^{-3}M) displayed mainly three peaks, the first and second peaks were observed at 224 nm and 263 nm were assigned to the moderate energy π-π^* transition of the aromatic rings. The third peak (λ_{max}) was observed at the 444 nm was referred to the π-π^* transition of intermolecular charge- transfer taken place from benzene through the azo group (-N=N)[19].

2. Invitro enzymic activity

The effects of azo compound on serum cholinesterase activity in vitro were carried out. Some of this compound showed encouraging inhibitors as dibucaine and Naf[25]. The centage of inhibition of these compounds are shown in table(1, 2, 3).

Table(1):- The effect of different concentrations of 4,4-azo dibenzoic acid on AchE activity.

inhibitor Conc.(M)	Enzyme activity µmol / ml / 3min	Inhibition %	Recovery %
Nil	4.5 ± 0.19	0.0	100
4.5 * 10^{-3}	3.01 ± 0.22	33.12	66.88
4.5 * 10^{-4}	3.27 ± 0.17	27.34	72.66
4.5 * 10^{-5}	3.61 ± 0.30	19.78	80.22
4.5 * 10^{-6}	3.63 ± 0.18	14.34	80.66
4.5 * 10^{-7}	3.94 ± 0.29	12.45	87.55
4.5 * 10^{-8}	4.10 ± 0.31	8.89	91.11

Table (2):-The effect of different concentrations of 4-(4-Nitro benzene azo)-3-amino benzoic acid on AchE activity.

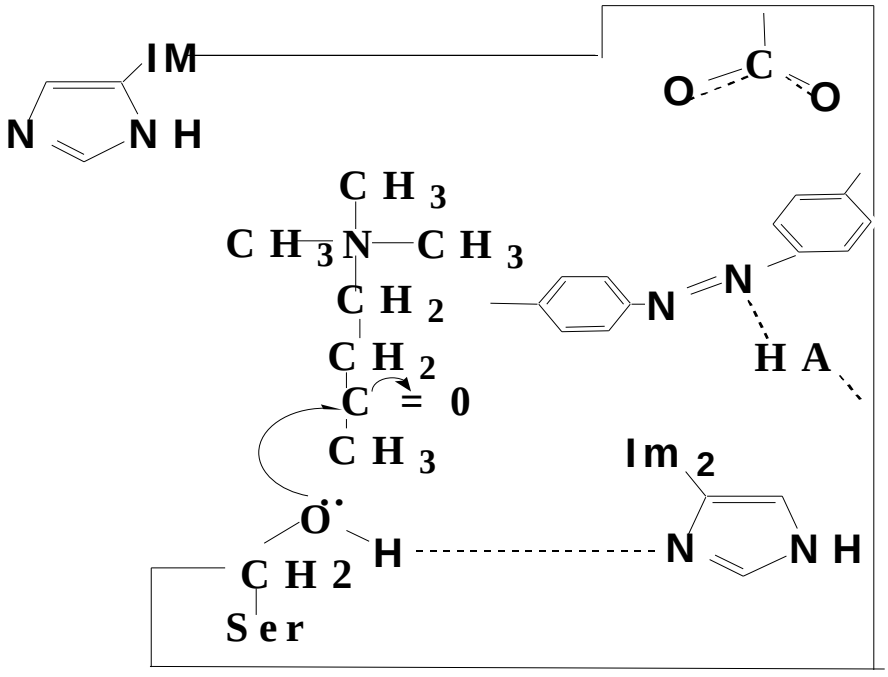
inhibitor Conc.(M)	Enzyme activity µmol / ml / 3min	Inhibition %	Recovery %
Nil	6.03 ± 0.52	0.0	100
4.5 * 10^{-3}	2.41 ± 0.12	60.04	39.96
4.5 * 10^{-4}	2.11 ± 0.31	65.01	34.99
4.5 * 10^{-5}	3.18 ± 0.18	47.27	52.73
4.5 * 10^{-6}	3.50 ± 0.19	41.96	58.04

4.5 * 10 $^{-7}$	4.25 ± 0.23	29.52	70.48
4.5 * 10 $^{-8}$	5.30 ± 0.27	12.11	87.89

Table (3):- The effect of different concentrations of 5-(2-Benzioc acid azo)-8-hydroxyquinoline on AchE activity.

inhibitor Conc.(M)	Enzyme activity µmol / ml / 3min	Inhibition %	Recovery %
Nil	5.71 ± 0.33	0.0	100
4.5 * 10 $^{-3}$	1.21 ± 0.27	78.99	21.01
4.5 * 10 $^{-4}$	1.53 ± 0.18	73.21	26.79
4.5 * 10 $^{-5}$	1.75 ± 0.32	69.36	30.64
4.5 * 10 $^{-6}$	1.91 ± 0.19	66.36	33.45
4.5 * 10 $^{-7}$	2.04 ± 0.30	64.28	35.72
4.5 * 10 $^{-8}$	2.54 ± 0.28	55.52	44.48

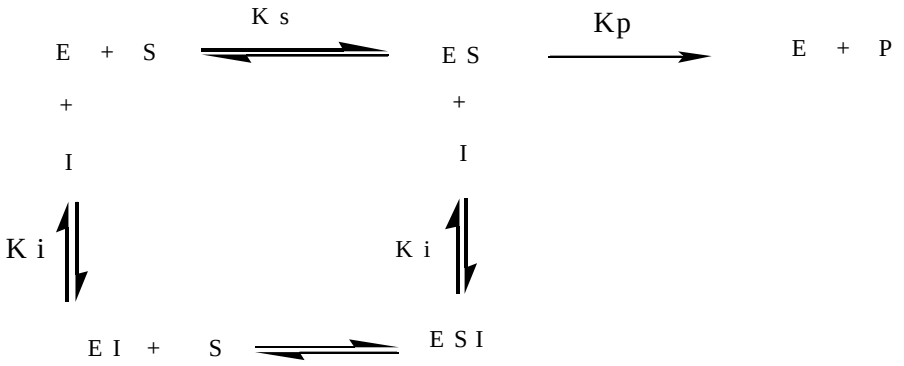
The variation in inhibition percentage of intend compounds may be attributed to the nucleophility of nitrogen atoms in these compounds this mean that these compound have pair of electron and behave as base to accept H from active side in the mechanism of hydrolysis of enzyme (CHE), OH^- group of serine as strong nucleophile to attack the (C=O) group of ester choline, and at the same time these compounds accept hydrogen H-A for Tyrosine to connect with, therefore prevent its coordination with oxygen of (C=O)(CHE), This make its electrophility C of (C=O) weak towards OH^- of serine, according this inhibited the enzyme activity[26].



Suggested chart

Kinetic of Inhibition

Type of inhibition was determined by line weaver–Burk plot, fig(1,2,3) which indicate that the inhibition proceeds as non competitive inhibition due to change in Vmax values while Km remained constant. The non competitive inhibition could be represented as follows [27-29].



Vmapp was calculated from

$$\frac{1}{V} = \frac{Km}{Vmax}\left[1 + \frac{I}{Ki}\right]\frac{1}{[S]} + \frac{1}{Vmax}\left[1 + \frac{I}{Ki}\right]$$

$$\frac{1}{Vmapp} = \frac{1}{Vmax.Ki}[I] + \frac{1}{Vmax}$$

$$\frac{1}{Vmapp} = \frac{1 + \frac{[I]}{Ki}}{Vmax}$$

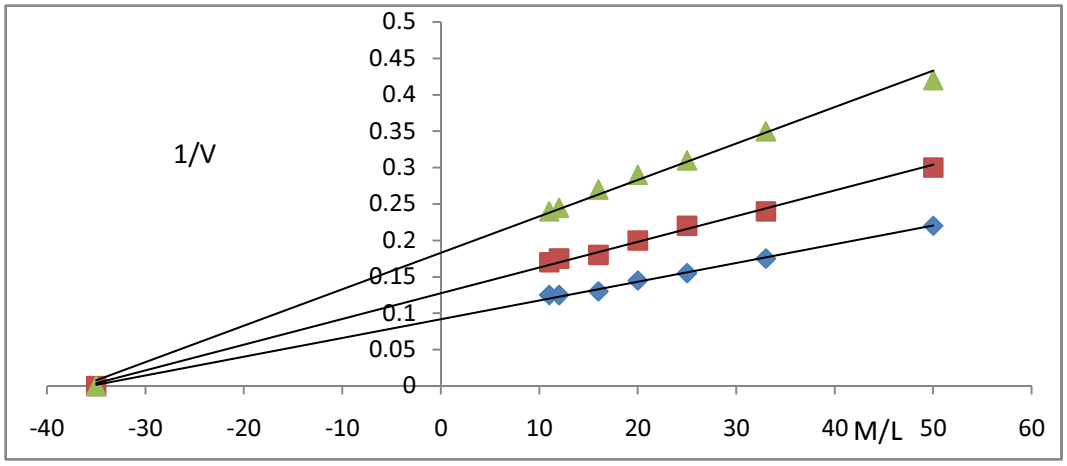


Fig.1 : lineweaver Burk polt determination Ki and Vampp values inhibition (AchE) (■)without (▲,■) with different concentration of compound (I) ($4.5 * 10^{-3}$, $4.5 * 10^{-8}$).

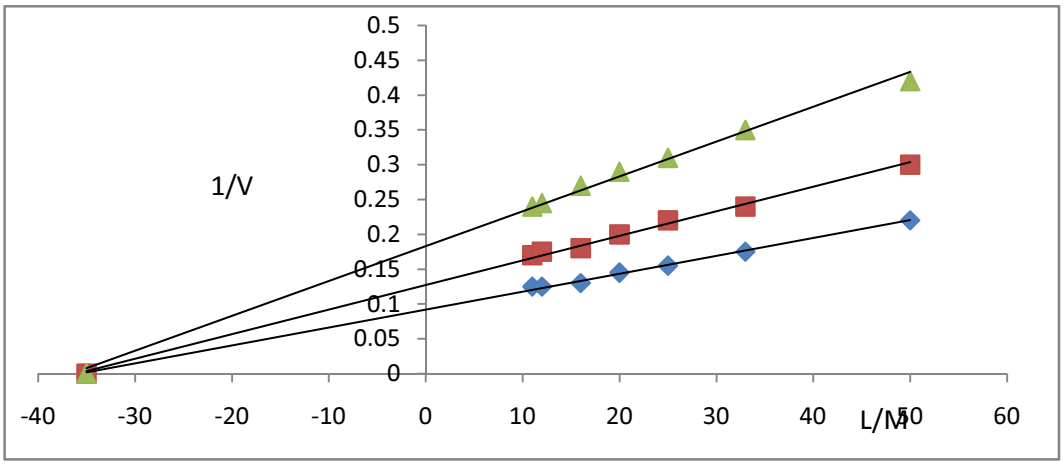


Fig.2 : lineweaver Burk polt determination Ki and Vampp values inhibition (AchE) (■)without (▲,■) with different concentration of compound (II) ($4.5 * 10^{-3}$, $4.5 * 10^{-8}$).

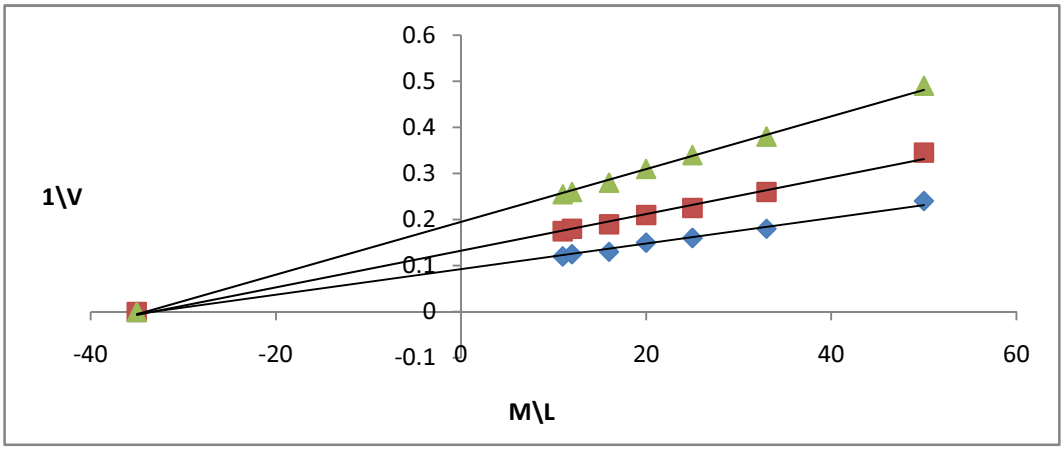


Fig.3 : lineweaver Burk polt determination Ki and Vampp values inhibition (AchE) (■)without (▲,■) with different concentration of compound (III) ($4.5 * 10^{-3}$, $4.5 * 10^{-8}$).

Table (4):-kinetic properties of AchE using line weaver – Burk polt.

No. of comp.	Conc. Of Inh. (M)	Vmapp µmol / ml / 3min	Ki (M)
I	$4.5 * 10^{-8}$	7.40	**$6.52 * 10^{-8}$**
II	$4.5 * 10^{-8}$	8.00	**$3.3 * 10^{-8}$**
III	$4.5 * 10^{-8}$	11.11	**$3.41 * 10^{-8}$**

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