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Purification and kinetic properties of hepatic γ-glutamyltransferaseenzyme from sarcocystosis-infested buffalo's liver

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ABSTRACT

Background: γ-glutamyltransferase(EC: 2.3.2.2) activity increased in hepatocellular diseases in animals and human including sarcocystosis. ***Objectives:*** The present study aimed to investigate the activities of hepatic enzyme; alanine transaminase (ALT), aspartate transaminase (AST), and gamma glutamyltranspeptidase (GGT) during the sarcocystosis infestation in buffalo. Also, to explore the purification, kinetic properties, and effect of anticoccidial drug ambrolium on the most affected enzyme. ***Methods:*** Blood and liver samples were collected from 10 sarcocyst-free and 15 sarcocyst-infested buffalos from Alexandria abattoirs, Egypt. ***Results:*** The result revealed that the specific activities of plasma ALT and AST were non-significantly increasedbut, GGT significantly increased in infested buffalo. Hepatic ALT, and GGT specific activities were significantly decreased while, AST activity was increased in infested-buffalo. The purified GGT enzyme had lowered specific activity in infested buffalo than control one and the enzyme consist of two subunits with molecular weight of 29.000 and 70.000 dl. Also, the purified enzyme from infested-buffalo had higher K_m and lowered V_{max} as compared to control one. The optimum temperature of purified GGT was 37 ºC and the ambrolium drug had an inhibitory effect on purified enzyme. ***Conclusion:*** We concluded that, the alteration in hepatic enzymatic activities considered a biomarker for diagnosis of sarcocystosis in buffalo, and ambrolium had an inhibitory effect of competitive type on purified enzyme

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1. Introduction

Sarcocystosis is an important food-borne parasitosis in humans and various animals [(1)]. It is one of the emerging diseases of livestock in recent time; it has been recognized as a distinct disease entity [(2)]. Bovine sarcocystosis is an economically important disease worldwide caused by the apicomplexan protozoan, sarcocystisspecies [(3)]. Dessouky et al. [(4)] reported that buffalo calves infested with sarcocystisfusiformissporocysts had minor change on ALTactivity. Furthermore, Prasse and Fayer [(5)] found that lactate dehydrogenase (LDH) and AST activities were high in sarcocystisbovicanissporocysts infested calves. Also, Phillips and Ford [(6)] demonstrated that lambs inoculated with sarcocystistenellasporocysts had elevated AST activity.

The activities of blood serum enzymes; creatine kinase (CK), GGT, and LDH were investigated in seven calves during the course of experimental sarcocystosis. In calves infested orally with sarcocystiscruzisarcocysts, these enzyme activities differed from those found in control animals. Catalytic activity of GGT increased during the third week post infestation, with a maximum in the 9^{th} week. Interestingly, in calves infested with the lowest doses of sporocysts, the catalytic activity of this liver enzyme was increased as soon as one week after infestation [(7)].

The aim of the present study was undertaken to throw the light on biochemical investigation the biochemical changes in hepatic enzymes (ALT, AST and GGT) in sarcocystosis-infested buffalo. Purification, kinetic studies and effect of anticoccidial drug were involved for the most affected enzyme (GGT).

2. MATERIAL AND METHODS

2.1. Chemicals

DEAE-cellulose was purchased from FlukaChemie Co., Germany. Deoxycholic acid was purchased from Sigma Co., Germany. Tris (hydroxy methyl methylamine) was purchased from Molekula, UK. Acrylamide, SDS, Coomassie Brilliant blue R-250 and glycine were purchased from Bio Basic INC., Canada. 2- β-mercaptoethanol was purchased from Merck-schuchardt., Germany. Tris-HCl and N, N-methylene-Bis-Acrylamide were purchased from Vivantis., Malaysia. Ammonium persulfate was purchased from LobaChemie., India. The kinetic assay kit for GGT was purchased from the Spectrum Co., Egypt. The kinetic assay kit for ALT, AST and the colorimetric assay kit for protein were purchased from N.S.BIO-TEC Co., Egypt. Methanol and ethyl alcohol was purchased from Chemajet Chemical Co., Egypt. Sodium hydroxide, sodium dihydrophosphate (NaH_2PO_4), disodium hydrophosphate ($NaHPO_4$), glacial acetic acid and ammonium sulfate were purchased from El-Nasr Pharmaceutical Chemical Co., Egypt.

2.2. Survey and sample collections

The samples were obtained from 10 different control buffalo carcasses and 15 different naturally infested buffalo carcasses from Alexandria abattoir. The post mortem examination of naturally infested buffalo carcasses revered the presence of macroscopic sarcocyst in tongue, esophagus, masseter muscle, skeletal muscles and occasionally heart.

2.3. Plasma and liver sampling

The blood samples were collected in EDTA tubes, centrifuged at 3.000 rpm for 10 minutes and the plasma supernatant of each blood sample were

separated, collected and stored at -20 ºC until determination of ALT, AST and GGT activities as well as protein content of each plasma sample according to manufacturer instructions. The specific activity of the enzyme of each sample was calculated by dividing the enzyme activity by protein content. Liver samples were collected and divided into two parts; the first part was weighed and homogenized by mortar in 9 volumes of 0.1 M sodium phosphate buffer, pH 7.4. Each homogenate was centrifuged at 4.000 rpm for 20 minutes. The supernatant for each sample was separated, collected and stored at -20 ºC until determination of ALT, AST and GGT activities as well as protein content of each liver sample. The specific activity of the enzyme of each sample was calculated by dividing the enzyme activity by protein content. The other part of each liver sample for each group was washed, weighed and exercised for purification as described in section 2.4

2.4. Purification of liver GGT enzyme

Purification of GGT enzyme from control and infested buffalo's liver samples was carried out as described previously according to Tsuchida et al**.** **(8)**. The liver samples were washed, weighted and cooled. The cold livers were homogenized by the mortar homogenizer in cold buffer (7 ml/g liver) of 10 mMTris-HCl (pH, 0.8) containing 150 mMNaCl. The homogenate was centrifuged at 2000 rpm for 10 min. The supernatant was centrifuged at 12.000 rpm for one hour, and then the precipitate (pellet) was collected. The precipitate was treated with six volume of 0.5% Triton X-100 in 10 mMTris-HCl (pH, 0.8) containing 150 mMNaCl for two hours at room temperature with shaking slowly, and then centrifuged at 6.000 rpm for half hour and the supernatant was collected. The supernatant was fractioned with 50% ammonium sulfate (31 g/100 ml supernatant), then centrifuged at 6.000 rpm for half hour and the precipitate (pellet) was collected. The dialyzate was treated with 1% deoxycholic acid in 10 mMTris-HCl with shaking slowly in cold condition for half hour, then centrifuged at 6.000 rpm for half hour and the supernatant was collected. The supernatant was fractioned with 50% ammonium sulfate (31 g/100 ml supernatant), then centrifuged at 6.000 rpm for half hour and the pellet was collected. The pellet was dialyzed against 10 mM sodium phosphate buffer, pH 7.5. The dialyzed buffer was changed every two hour and keep overnight. Finally, the dialyzate was applied onto a DEAE-cellulose column (7 cmX 1 cm) which was equilibrated by the same dialyzing buffer. GGT was not absorbed and was collected in the void volume. The enzymatic activity and protein concentration of each step was determined and the specific activity was calculated.

2.5. SDS polyacrylamide gel electrophoresis

SDS polyacrylamide gel electrophoresis was applied for each step of purification of GGT enzyme at 10% polyacrylamide concentration according to the method described bySchagger **(9)**.

2.6. Protein assay

Determination of protein was based on the production of colored complexes between peptide bonds and cupric ions in alkaline medium **(10)**.

2.7. The kinetic study on purified buffalo GGT

2.7.1. Effect of substrate concentration

The different concentrations of L-γ-Glutamyl-3-carboxy-4-nitronilide substrate (0.0, 200, 400, 500, 800, 1000 Mm) in the presence of glycylglycine were prepared and then mixed with appropriate amounts (100 µL) of purified GGT from control and infested buffalo liver samples. The GGT enzymatic activity was assayed as described above for different substrate concentrations to study its effect. The specific activity as an initial velocity (*v*) was calculated by dividing the value of enzyme activity by the protein content. These values were used for drawing the saturation curve by plotting the initial activity (*v*) versus the corresponding substrate concentration [S]. Also, Lineweaver-Burk plot (1/*v* versus 1/[S]) was drawn as a reciprocal plot by the reciprocal values of *v* and [S]. The values of kinetic parameters Michaelis constant (K_m) and maximum velocity (V_{max}) were determined.

2.7.2. Heat stability of GGT from control and infested buffalo hepatic samples

GGT from control and infested bovine hepatic samples was treated with different temperature values (37, 40, 50, 60, 70 ºC). The appropriate amounts (100 µL) of heat treated enzyme were used to assay GGT enzymatic activity to study the heat stability of the enzyme. The specific activity was calculated by dividing the value of enzyme activity by the protein content. The results were tabulated and curve was drawn by plotting the specific activity versus the corresponding temperature.

2.7.3. Effect of Antimicrobial drug ambroliumon purified buffalo GGT

The ambrolium drug (20%) was diluted 10 times before use and different concentrations were prepared (0.0, 3.25, 6.5, 9.75, 13.0, 16.25, and 19.5 mM). The appropriate amounts (100 µL) of purified GGT from control and infested bovine liver samples were mixed with different concentrations of the drug at two substrate concentrations (500, 1000 mM). GGT enzymatic activity was then kinetically assayed to study the drug effect. The specific activity as an initial velocity (v) was calculated by dividing the value of enzyme activity by the protein content. The results were tabulated and reciprocal plots were drawn by plotting the reciprocal values of V versus the corresponding ambrolium concentrations at two substrate concentrations.

2.8. Statistical analysis

The specific activities of ALT, AST, and GGT enzymes in different plasma and liver samples from control and infested buffalo were statistically analyzed by student's t-test.

3. RESULTS

3.1. Specific activities of ALT, AST, and GGT in control and infested buffalos

The data represented in table (1) showed that the specific activities of plasma ALT and AST non-significantly increased while, plasma GGT specific activity was significantly increased in sarcocystosis infested buffalo as compared to control buffalo. The specific activities of hepatic ALT, and GGT were significantly decreased in sarcocystosis infested buffalo when compared to control buffalo. On the other hand, hepatic AST specific activity was increased in sarcocystosis infested buffalo when

compared to the values in control healthy buffalo group.

3.2. Purification of hepatic GGT from control and infested buffalo

As shown in table (2,3), the GGT enzyme from hepatic samples from control buffalo was purified with a specific activity 0.034 by DEAE-cellulose column chromatography with a yield of 6.08. The data represented in table (3) showed that GGT enzyme from hepatic samples from infested buffalo was purified with a specific activity 0.024 by DEAE-cellulose column chromatography with a yield of 5.2. the purification was run out into six steps; triton x-100 extract, 50% ammonium sulfate precipitation, deoxycholic acid extract, 50% ammonium sulfate precipitation, and ion-exchange chromatography (DEAE-cellulose column).

3.3. SDS polyacrylamide gel electrophoresis for purified hepatic GGT from control and infested buffalo

SDS polyacrylamide gel electrophoresis was made for different steps of purification of hepatic GGT from control and infested buffalos at 10% polyacrylamide gel (after DEAE cellulose column step) showed that the purified hepatic GGT consisted of two subunits with molecular weight of 29.000 and 70.000

3.4. Kinetic studies on purified buffalo GGT from control and infested buffalos

3.4.1. The effect of substrate concentration on purified GGT

The effect of substrate concentration on purified GGT showed on increasing the substrate concentration from 0.0 to 1000 mM, the specific activity as aninitial velocity v_0 (U/mg) was increased from 0.0 to 0.028 for the control-purified buffalo GGT while it was increased from 0.0 to 0.024 for the infested purified buffalo GGT. The kinetic parameters of total GGT showed that the values of K_m and V_{max} were 4.396 mM and 6802.7 U/mg, respectively of control buffalo GGT, while these values were 14.81 mM and 2020.2 U/mg, respectively of infested buffalo GGT as showed in figure (1).

3.4.2. Heat stability of the hepatic buffalo GGT

The heat stability of hepatic buffalo GGT showed that with increasing of temperature from 37 to 70 ºC, the specific activity (U/mg) of control buffalo GGT was decreased from 7.75 X 10^{-3} to 0.36 X 10^{-3}. The specific activity (U/mg) of infested buffalo GGT was decreased from 7.05 X 10^{-3} to 0.36 X 10^{-3} with increasing temperature from 37 to 70 ºC. The optimum temperature for hepatic buffalo GGT was 37 ºC, at which the specific activity of hepatic buffalo GGT from control was 7.75 X 10^{-3} and from infested was 7.05 X 10^{-3}. The enzyme from the infested buffalo is less stable at 60 ºC compared to that from control (Fig. 2).

3.4.3. *In vitro* effect of ambrolium drug on purified GGT from control and infested buffalos

The effect of ambrolium on purified GGT from control buffalo was studied kinetically by Dixon plot; figure (3) showed an inhibitory effect of competitive type inhibition as the two lines intersect between the two axes. At substrate concentration of 500 mM and increasing ambrolium concentration from 0.0 to 19.5 mM, the specific activity (U/mg) decreased from 0.015 to 0.002.

At substrate concentration of 1000 mM and increasing ambrolium concentration from 0.0 to 19.5 mM, the specific activity (U/mg) decreased from 0.028 to 0.013. The effect of ambrolium on purified GGT from infested buffalo was studied kinetically by Dixon plot; figure (4) showed an inhibitory effect of competitive type inhibition as the two lines intersect between the two axes. At substrate concentration of 500 mM and increasing ambrolium concentration from 0.0 to 16.25 mM, the specific activity (U/mg) decreased from 0.014 to 0.009. At substrate concentration of 1000 mM and increasing ambrolium concentration from 0.0 to 19.5 mM, the specific activity (U/mg) decreased from 0.024 to 0.0075.

4. DISCUSSION

The present study was focused on biochemical investigation of hepatic enzymes; ALT, AST, and GGT in buffalo sarcocystosis. The elevation of plasma ALT is regarded as an indicator of liver damage based on the presumption that ALT protein is specifically and abundantly expressed in the liver [(11)]. It is possible that sarcotoxin participates in damage of liver cells [(7)]. Therefore, leakage of ALT, AST, and GGT from damaged liver is the probable cause of increased plasma and decreased hepatic enzyme activity. The non-significant increase of plasma AST specific activity was in agreement with that reported by Dessouky et al.[(4)].

The data of the present study showed that the yield of GGT purification from control or infested buffalo's liver is weekindicated by the faint bands with the following steps of hepatic GGT purification were an indication for low protein concentration of the step.

SD-PAGE of purified hepatic GGT from control and infested buffalos at 10% polyacrylamide gel showed that the purified hepatic GGT consisted of two subunits with molecular weight of 29.000 and 70.000 dl. The obtained results agreed with Furukawa et al.[(12)] who reported that GGT was purified to a homogenous state from bovine liver. It had an apparent molecular weight of about 110.000, as judged by polyacrylamide gel electrophoresis and consisted of non-identical glycopeptides with molecular weight of 68.000 and 27.000 dl. During DEAE-cellulose chromatography, liver GGT from rats was adsorbed at 0.1M Tris-HCl buffer (pH8.0). The Km value of the liver GGT for L-gamma-glutamyl p-nitroanilide was 1.35mM [(13)].

Our result regarding the heat stability of hepatic GGT from control and infested buffalo showed that the optimum temperature of hepatic GGT was 37 ºC. The enzyme from infested buffalo is less stable at 60 ºC compared to that from control. GGT was purified from Triton X-100 solubilized neutrophils and its kinetic parameters were determined. The neutrophil GGT was incubated at 56 ºC at different time interval (5-30 min) keeping all other variables constant for the thermal stability determination. For optimum temperature detection, the GGT activity was determined after incubation for one minute at different temperature (25-56 ºC). The optimum temperature was 37 ºC. It had thermal stability with 58% relative activity at 56 ºC for 30 min incubation [(14)].

5. CONCLUSION

The present study revealed that infestation of buffalo with sarcocystosis

increased liver enzymatic activities and the most affected is GGT enzyme that competitively inhibited by ambrolium drug.

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Table (1): Specific activities of plasma and hepatic ALT, AST, and GGT enzymes in control and infested buffalo

	ALT	AST	GGT
Control plasma (mU/mg)	46.33±2.9	161.1±14	10.37±0.4
Infested plasma (mU/mg)	56.67±3.7	178±11.5	16.77±1.9*
Control hepatic (U/mg)	1.04±0.07	1.02±0.15	0.85±0.06
Infested hepatic (U/mg)	0.21±0.02**	1.74±0.15*	0.3±0.03**

Values are expressed as mean ± S.E
* Significant at P<0.05 ** highly significant at P<0.00

Table (2): Purification of hepatic GGT from control buffalo

Step	Total activity	Total protein	Specific activity	Yield	Fold
Homogenate	4.261	1352.4	0.0031	100	1
Triton extract	0.469	115.7	0.004	11.01	1.29
Post 50% $(NH_4)_2SO_4$ P.P.T	0.602	48.0	0.0125	14.24	4.03
Deoxycholic acid extract	1.084	93.6	0.011	25.81	3.54
Post 50% $(NH_4)_2SO_4$ P.P.T	0.333	17.8	0.0187	7.81	6.03
Post DEAE cellulose column	0.259	7.5	0.034	6.08	10.06

Table (3): Purification of hepatic GGT from infested buffalo

Step	Total activity	Total protein	Specific activity	Yield	Fold
Homogenate	3.385	1152.4	0.0029	100	1
Triton extract	0.873	127.3	0.007	25.8	2.41
Post 50% $(NH_4)_2SO_4$ P.P.T	0.669	58.7	0.0114	19.76	3.93
Deoxycholic acid extract	0.984	120.0	0.0082	29.07	2.83
Post 50% $(NH_4)_2SO_4$ P.P.T	0.231	14.4	0.0160	6.8	5.52
Post DEAE cellulose column	0.178	7.5	0.024	5.2	8.27

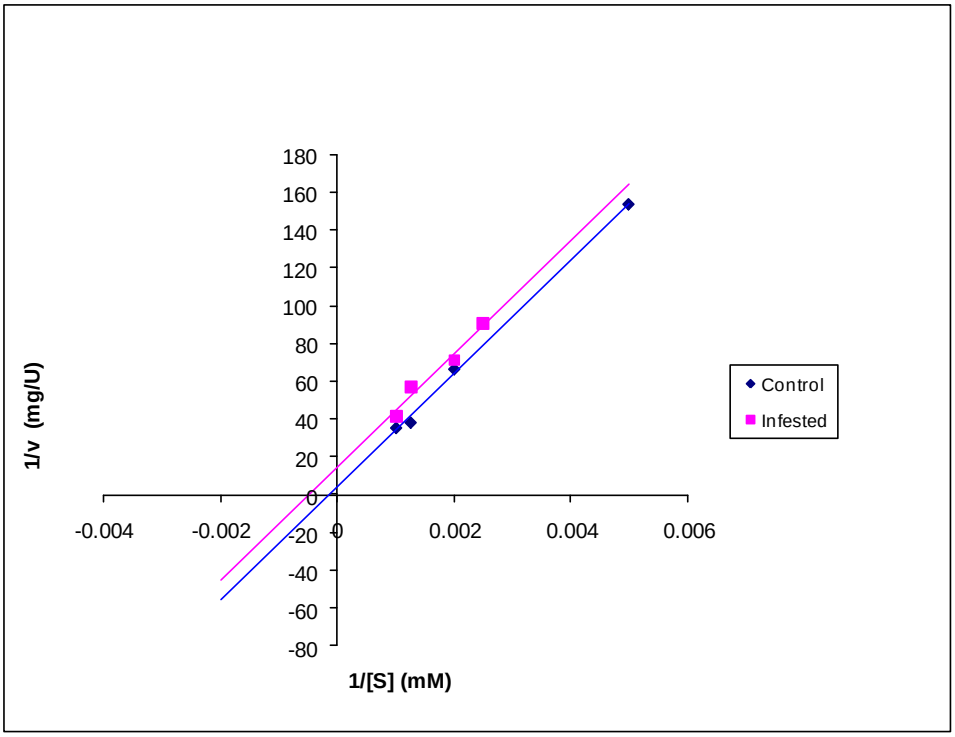

Figure (1): Reciprocal plot of purified hepatic GGT from control and infested buffaloes

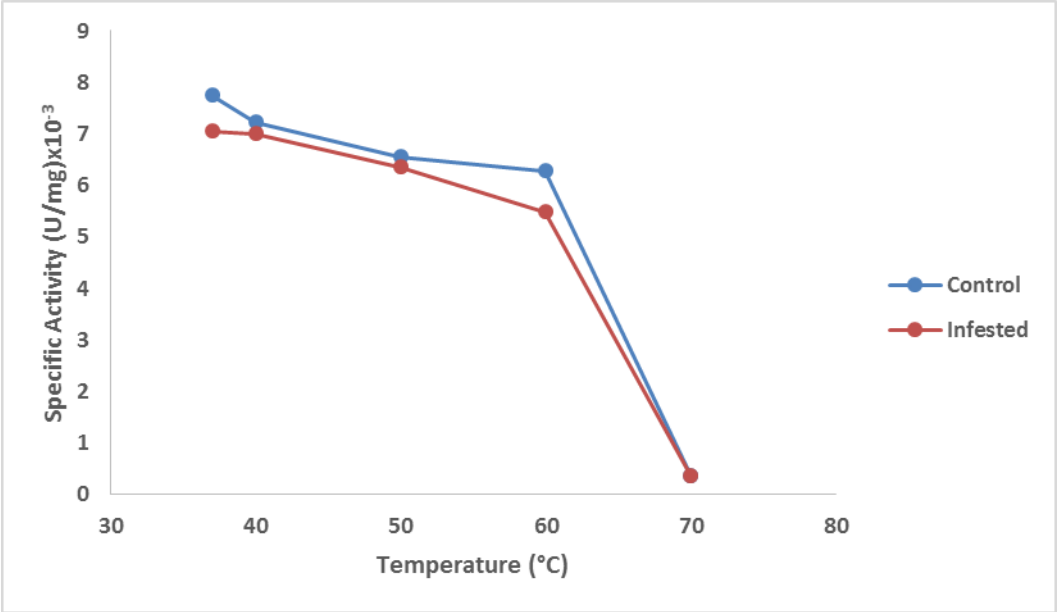

Figure (2): Heat stability of hepatic buffalo GGT from control and infested buffalo

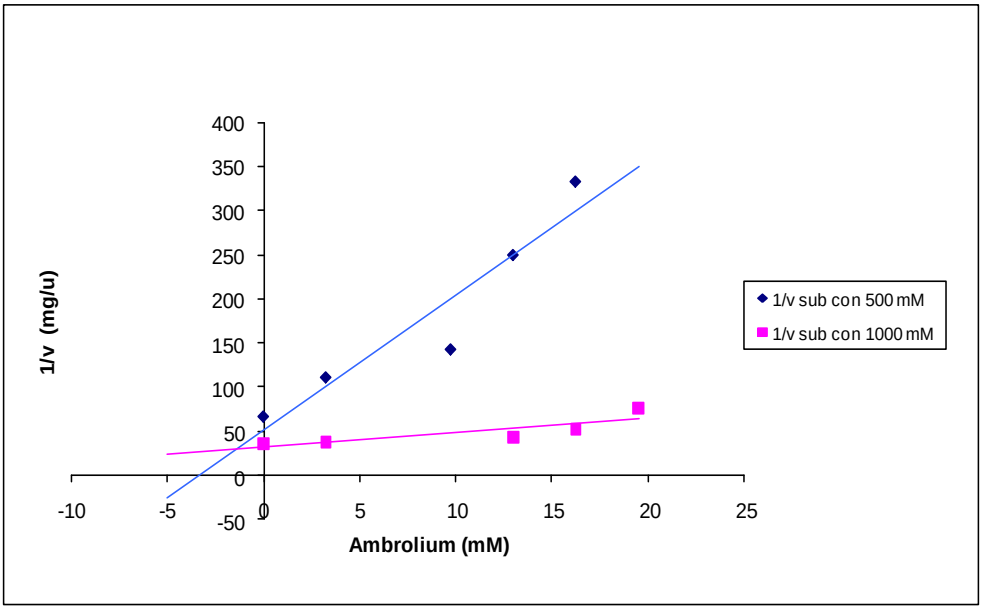

Figure (3): ***In vitro*** **effect of ambrolium on purified hepatic GGT from control buffaloes.**

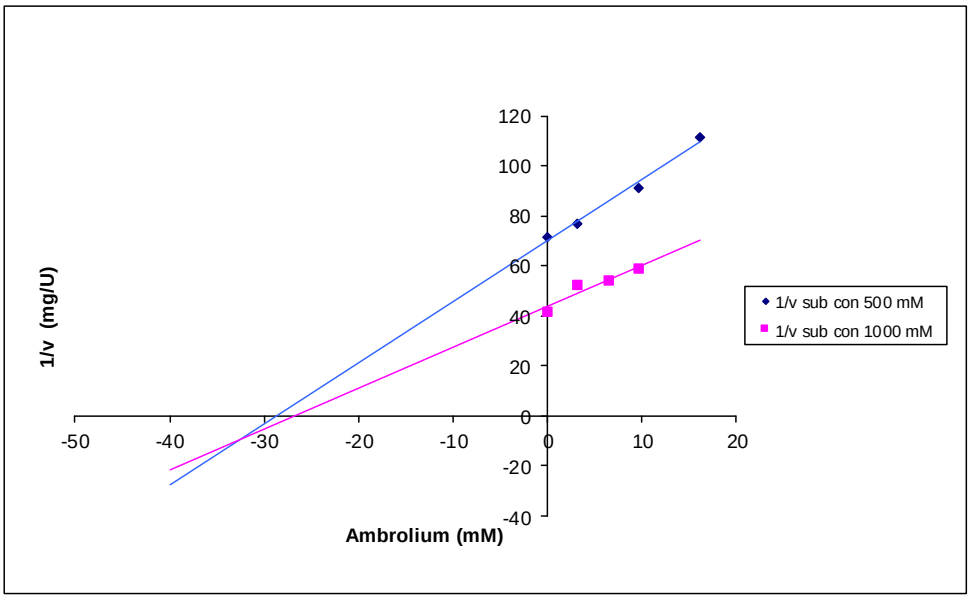

Figure (4): ***In vitro*** **effect of ambrolium on purified hepatic GGT from infested buffaloes.**