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Impact of anthelmintic efficacy of *Calotropis procera on* tegumental enzymes of the trematode, *Gastrothylax indicus*

Rama Aggarwal[1], Upma Bagai[2]
[1]*Parasitology Laboratory, Department of Zoology Panjab University, Chandigarh-160014,India.*
[2]*Parasitology lab, Department of Zoology, Panjab University, Chandigarh-160014,India.*

Abstract: *The trematode parasite, Gastrothylax indicus was exposed to ethanolic and aqueous flower extract of Calotropis procera to evaluate the anthelmintic efficacy of the plant. The parasites were incubated in 6.25, 12.5, 25, 50 mg ethanolic and aqueous extracts per ml of PBS at a temp of 37± 1ºC. Mortality was observed at 0.5± 0.05h and 0.75± 0.10h for ethanolic and aqueous extracts respectively for the parasite at the highest test concentration of the plant extract. The commercial anthelmintic albendazole was tested for various concentrations ranged from 20-80 µg/ml and mortality was observed instantly (0.08±0.01h) at the concentration of 80 µg/ml. To further investigate the efficacy of plant extract, vital tegumental enzymes of the parasite viz. Alkaline phosphatase (ALP), Acid phosphatase(ACP), Adenosine triphosphatase (ATPase) and Glucose- 6-phosphatase (G-6-pase) was found to be suppressed by 43.890, 30.287,18.970 and 22.842% by ethanolic extract and 62.710, 19.780, 57.554 and 10.035% by aqueous extract whereas albendazole inhibited 41.617, 25.650, 64.797 and 26.611% respectively. Enzyme kinetic studies showed inhibition to be non-competitive in case of ACP with both the extracts and albendazole whereas for ALP it was found to be non- competitive with ethanolic and mixed type with aqueous extract. Albendazole showed competitive inhibition in case of ALP.*

Keywords*- Anthelmintic, Calotropis procera, Gastrothylax indicus, Tegumental enzymes*

I. INTRODUCTION

Helminthic infestation is a major health problem world-wide more particularly in third-world countries because of its prevalence there. However, several serious long-term complications result as after effect of such infection. Thus, iron-deficiency anaemia, malnutrition, dysentry and rectal prolapsed are some of the manifestations of the helminthes like hookworms, *Ascaris* and *Trichuris*. Sometimes, helminth-infected persons have been reported to suffer from other infections like tuberculosis, HIV and malaria due to suppression of immune response caused by different helminthes [1]. *Gastrothylax indicus* (Class: Trematoda) and phylum Platyhelminthes, are responsible for paramphistomosis characterized by acute parasitic gastroenteritis with high morbidity and mortality rates [2,3,4]. A number of drugs have been assessed and recommended for the treatment of paramphistomosis [2,5].

Calotropis procera known as aak, is used in ethnoveterinary medicine system as an expectorant, anthelmintic, laxative, purgative, anti-inflammatory and diuretic [6]. Different parts as well as latex of *C. procera* have been reported to have emetic, purgative and anthelmintic effects in traditional medicine [7]. *C. procera* flowers possess good anthelmintic activity against nematodes [6].

Phosphatases are known to play a variety of important roles at the transporting surfaces [8,9], in extracellular digestion and phosphorylation of nutrients transported, secreted and excreted [10]. Due to their presence at the absorptive surfaces, phosphatases react with the substances in the external milieu [9]. Anthelmintics may alter the enzymes and modify the normal metabolism of the absorptive surfaces during their absorption. In this work, the *in vitro* effect of ethanolic and aqueous extract of *C. procera* and albendazole on phosphatases was studied in *Gastrothylax indicus*, in order to get a better in sight into the complex enzyme substrate interaction and the mechanism of inhibition of the enzyme by the extracts.

II. MATERIALS AND METHODS

2.1 Plant material

Flowers of aak (*Calotropis procera*), were collected from in and around Chandigarh. The plant material was identified in Department of Botany, Panjab University, Chandigarh with Voucher number- 4830.

2.2 Preparation of extracts

Flowers of *C.procera* were washed thoroughly, shade dried and grounded by motor driven grinder into powder form. Both ethanolic and aqueous plant extracts were prepared [6]. Ethanolic flower extract of *C.procera* (EFECP), was exhaustively extracted by mixing 80 gm of powdered plant material and adding

approximately 300ml of ethanol in a soxhlet apparatus. Aqueous extract (AFECP) was prepared by dissolving 100gm of powdered plant material mixed with 500ml of distilled water in 1L flask and boiled for 4-6 h in water bath. It was allowed to macerate at room temperature for 24 h and the brew was filtered through muslin gauze and Whatman filter paper No.1. Both ethanolic and aqueous extracts of plant material were evaporated in Rota evaporator to give crude ethanolic and aqueous extracts. The extracts were scraped off and transferred to screw capped vials at -4°C until used.

2.3 *In vitro* experiment

Worm motility inhibition assay was employed for the evaluation of anthelmintic activity of EFECP and AFECP under *in vitro* conditions [11]. The *in vitro* anthelmintic activity was carried out on adult *G.indicus* worms to determine the inhibitory effect of extracts on adult worms. Mature *G. indicus* were collected from the rumen of sheep/goat procured from slaughter house. The worms were washed in phosphate buffered saline (PBS pH7.2) and finally suspended in PBS. The study was conducted at four different dilutions of both the extracts viz., 6.25, 12.5, 25, 50, mg/ml prepared in PBS. The crude aqueous extract was diluted in PBS, whereas, crude ethanolic extract in 1%DMSO in PBS. Albendazole dissolved in 1% DMSO and diluted in PBS at concentrations of 20, 40, 60, 80 µg/ml and PBS alone served as postitive and negative control respectively. There were three replicates for each treatment concentration.Immediately after the mortality ,the parasites incubated in 6.25 mg of plant extracts per ml of PBS were selected for biochemical studies. For albendazole selected concentration was 20 µg/ml.

2.4 Biochemical analysis

Treated parasites and their respective controls were retrieved from the incubation media at the time when paralysis was seen and were processed for enzymatic analysis. A 10% homogenate of the enzymes in their respective buffers or 0.25M sucrose was centrifuged at 5,000 rpm for 25 min at 0ºC. The supernatant served as source of enzyme activity for different enzyme assays.

2.4.1 Alkaline phosphatase (ALP) estimation

The activity of alkaline phosphatase was determined by using p-nitrophenyl phosphate which gets hydrolyzed by action of phosphatase in alkaline pH to *p*-nitrophenol [12].
1 ml of buffered substrate (5.5 mM *p*-nitrophenol phosphate in 0.05 M glycine-sodium hydroxide buffer, pH 10.5) was incubated at 37ºC for 5 min to pre equilibrate. After this 0.1 ml of enzyme was added and incubated again for 15 min at 37ºC. Then 5 ml of 0.085N sodium hydroxide was added to stop the reaction and the liberation of p-nitrophenol was measured at 420nm. 100 µmol/ml *p*-nitrophenol was used as working standard and the optical density of all the tubes was read at 420 nm after 10 min

2.4.2 Acid phosphatase (ACP) estimation

The activity of acid phosphatase was determined by using p-nitrophenyl phosphate, which gets hydrolyzed by the action of phosphatases in acidic pH to *p*-nitrophenol [12].
0.8 ml of buffered substrate *p*-nitrophenyl phosphate was taken in different test tubes. Tubes were incubated for 10 min to attain a temperature of 37ºC. Thereafter, 0.1 ml of homogenate was added to it and incubated again for 30 min at 37ºC. Then 5 ml of 0.1N sodium hydroxide was added to stop the reaction and the liberation of *p*-nitrophenol was measured. The optical density of all the tubes was read at 420 nm after 10 min.

2.4.3 Glucose-6-phosphatase (G-6-pase) estimation

The enzyme activity was measured in terms of inorganic phosphate formed from incubating medium. To 0.1 ml of homogenate, 0.3 ml of 0.1M citrate buffer pH 6.2, 0.5 ml of G-6-Pase and 0.1 ml of distilled water were added. These contents were incubated at 37ºC for 1 h. Then 10 ml of 10% TCA was added and the mixture was allowed to stand for 10 min. The mixture was then centrifuged at 2,500 rpm for 10 min. 1ml of the supernatant after centrifugation was taken and 0.5 ml of acid ammonium molybedate, 0.2 ml of ANSA and 7 ml of distilled water was added to it. This mixture was heated in a boiling water bath, cooled and read at 600 nm [13].

2.4.4 Adenosine triphosphate (ATPase) estimation

The reaction mixture contained 0.2 ml of 0.2 M Tris HCl buffer pH 7.6, 0.2 ml of 5 Mm $MgCl_2$, 0.2 ml of 10% homogenate and 0.2 ml of 5mM ATP solution. The mixture was incubated for 30 min at 37ºC. 1 ml of 10% TCA was added to it and it was allowed to stand for 10 min, centrifuged at 2,500 rpm for 10 min. 1 ml of supernatant was taken out and 0.5 ml of acid ammonium molybedate, 0.2 ml of ANSA and 7 ml of distilled water was added. The mixture was heated in boiling water bath, cooled and read at 600 nm [14].

2.4.5 Calculations

$$\text{Activity of Enzyme} = \frac{\text{O.D. of test} - \text{O.D. of blank} \times \text{Vol. of std.} \times \text{Conc. of std.}}{\text{O.D. of std.} - \text{O.D. of blank} \times \text{Incubation time} \times \text{Protein conc.}}$$

2.4.6 Specific activity

Specific activities of the enzymes were expressed as the units of enzymes activity per mg protein. Protein contents of different samples were estimated [15].

2.4.7 Enzyme kinetic studies

Vmax (maximum of apparent initial enzyme velocity) and Km (substrate affinity constant) of the ALP and ACP were determined by assaying the enzymes at different substrate concentrations [16]. Similarly, Vmax and Km of the enzymes were determined of the treated parasite with EFECP and AFECP as a whole and albendazole *in vitro* system.

2.4.8 Statistical analysis

Statistical analysis were carried out by employing Graph pad software 3 and data was expressed as mean ± S.D. for each group. The statistical significance of inter group difference of various parameters were determined by unpaired student's *t* test. The comparisons were made between the treated groups and control group of parasites. Exploratory enzyme kinetics was done using Sigma plot 8.0 software and p value < 0.05 was termed as statistically significant value.

III. RESULTS

Following the exposure to different concentrations of the plant extract, the parasites contracted sharply for some time and then went into a relaxed state and continued in the same state till they attained a condition of flaccid paralysis which was followed by death. Table I shows the mortality of worms treated with various concentrations of EFECP, AFECP and albendazole. The controls survived for 9.2 ±0.23h. The treated parasites showed a steady decline in their mortality and survival time with exposure to ascending concentrations of the test dosage. Thus a dose dependent paralytic effect and subsequent loss of motility of the parasite by the extract was evident. Results of enzymatic analysis showing tissue activity, specific activity, percentage inhibition, p value and effect on Vmax and Km due to EFECP, AFECP and albendazole have been summarized in Table II and figs.1-6

In vitro incubation of *G. indicus* with EFECP, AFECP and albendazole inhibited the activity of enzymes. Varying degrees of inhibition was observed with EFECP, AFECP and albendazole. ALP activity was inhibited 43.890% by EFECP and 62.710% by AFECP (Table II). EFECP was showing non-competitive inhibition behavior as Vmax got lowered but Km remained unchanged (Fig. 1) whereas AFECP was found to be mixed type inhibitor as both Km and Vmax got altered (Fig. 2). Inhibition was found to be statistically extremely significant. With albendazole activity of ALP was reduced to 41.617% (Table II). Inhibition was found to be competitive for ALP (Fig. 3) as Km got increased in the presence of albendazole. ACP activity was reduced to 30.287% and 19.780% ($p < 0.0005$) with EFECP and AFECP respectively (Table II). Inhibition was non-competitive in both the extracts as only Vmax got changed (Fig. 4, 5). With albendazole inhibition was found to be 25.650% (Table II). It was non-competitive in nature as only Vmax got lowered (Fig. 6) and was statistically extremely significant (Table II).

G-6-pase activity was reduced 22.842% by EFECP and 10.035% by AFECP. Albendazole showed the inhibition of 26.611%. EFECP and AFECP inhibited the activity of ATP ase by 18.971% and 57.554% respectively. Albendazole showed the reduction of 64.797%. Inhibition was found to be extremely significant (TableII).

IV. Discussion

Different classes of anthelmintics are established to show profound effects on the physical activities, generally culminating into loss of mobility and mortality of helminth parasites in a dose dependent manner [17,18]. Following this basic technique several plants/plants parts such as *Allium sativun, Zingiber officinale, Curcurbita Mexicana* and *Ficus religiosa* [19], *Aretemisia brevifolia* [11] *Cardiospermum halicacabum* [20] *Fumaria parviflora* [21] have been reported as potent anthelmintics.

The results of the present study showed that *C. procera* flower has good anthelmintic potential. The mortality of the worms (*G.indicus*) was much faster in albendazole treatment than in EFECP and AFECP. It is in agreement with the findings of earlier workers on different helminth parasites [22,23].

Transcuticular diffusion is a common means of entry into helminth parasites for non nutrient and non electrolyte substance in helminth. It has also been shown that this route is predominant for uptake of major broad spectrum anthelmintics; benzimidazole, levamisole and ivermectin by different nematodes, cestodes, and trematode parasites as opposed to oral injestion [24]. *In vitro* anthelmintic activity of crude extracts of *Coriandrum sativum* against *Haemonchus Contortus* was studied and found that hydro-alcoholic extract was better than aqueous extract due to easier transcuticular absorption of hydro-alcoholic extracts into the body of the parasite than aqueous extracts [25] .

Tegumental enzymes play a very important role in maintaining the tissue homeostasis within the parasite. Alkaline phosphatase take part in active transport through cellular membranes and acid phosphatase deals with intracellular digestion processes [26,27] . ATPase is known to be related to energy metabolism, active transport and lipid synthesis [28].G-6 Pase presumably has a role in digestion and in absorption of nutrients.

The mode of action of anthelmintics is diverse, reflecting the natural differences in the physiology of the parasite and its potential host. It has been firmly documented that one of the hallmark effects of any anthelmintic is the destruction of the worm's surface. It is due to the fact that the tegumental structures are primary parasite-host interfaces, vital for absorption of nutrients and perception of the surrounding micro environment provided by the host [29,30,31,18].

In the present investigation, enzyme activities in EFECP, AFECP and albendazole treated *G indicus* were found to be reduced significantly ($p< 0.05$) compared to the control ones. Other plant extracts such that of *Alpinia nigra* shoot-extract showed similar effect on ALP, ACP and ATPase activities of *Fasciolopsis buski* [32] . Inhibition in the activity of G-6 Pase in *Trichuris globulosa* was shown with ethanolic neem leaf extract [33] .

Enzyme kinetic studies showed inhibition to be non competitive in case of ACP with EFECP, AFECP and albendazole whereas for ALP it was found to be non competitive with EFECP and mixed type with AFECP. Non competitive nature of inhibition suggests that substrate binding to the catalytic site of enzyme was not affected. Competitive inhibition of ALP by albendazole shows that inhibitor because of structural similarity compete for the same substrate binding site of the enzyme.

Effect of ethanolic extract of neem leaves on the enzyme kinetics of glycogen phosphorylase, G-6- pase and ATPase in *Trichuris globulosa* was studied. Non competitive inhibition was reported for glycogen phosphorylase and ATPase whereas it was found to be mixed type for G-6- pase [33].

V. CONCLUSION

The effects of the test plant on the motility and survival of the parasite and inhibition caused in the tegumental enzymes clearly indicate that phytochemicals of *C.procera* flowers may act as potential vermifuge or vermicide. In view of these observations further biochemical studies involving isolated active component (s) of this plant are warranted to confirm its anthelmintic efficacy.

VI. ACKNOWLEDGEMENT

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Table I. *In vitro* effect of plant extracts (EFECP, AFECP) and Albendazole on G. indicus

Dose Plant extract(mg/ml)/ Albendazole (µg/ml)	**Time taken in hours for mortality of *G.Iindicus***			
	INCUBATION MEDIUM			
	EFECP	AFECP	ALBENDAZOLE	Control(PBS)
6.25/20	3.50±0.18	4.16 ± 0.35	3.25±0.12	9.2±0.23
12.5/40	2.0±0.15 0.15	2.8± 021	1.50±0.17	
25.0/60	1.0± 0-08	1.5± 0.15	0.50±0.02	
50.0/80	0.5± 0.08	0.75± 0.10	0.08±0.01	

Values are expressed as mean± S.D. Each test was done in triplicate.

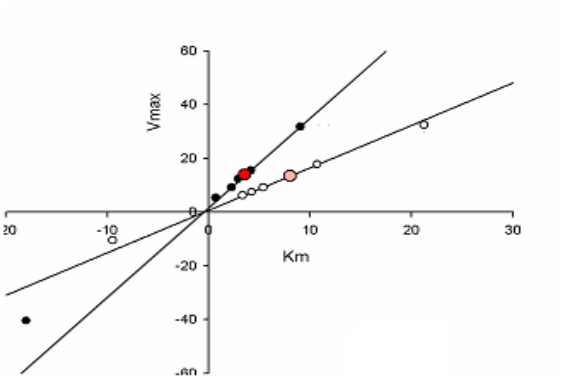
Table II. Effect of EFECP, AFECP and albendazole on tissue activity (units/g wet wt/min) and specific activity (units/mg protein/min) of ALP, ACP, G-6-pase and ATPase in *G. indicus in vitro*

Control/Treatment PBS/(mg/ml)	**ENZYME ACTIVITY (Tissue/Specific)**			
	ALP	**ACP**	**G-6-pase**	**ATPase**
Control	9.501± 0.157	34.488± 0.306	1.810±0.010	2.344±0.020
	5.00± 0.083	19.150± 0.162	0.952±0.005	1.244±0.011
	5.33± 0.09	27.750± 0.025	1.397±0.010	1.910±0.017

EFECP(6.25)	2.806±0.047	13.350± 0.208	0.735±0.005	1.008±0.009
%Inhibition/p	43.890%/p<0.0001	30.287%/p<0.0001	22.842%/p<0.0001	18.971%/p<0.0001
Control	7.761± 0.158 4.084± 0.083	34.485± 0.306 18.150± 0.162	1.810±0.010 0.952±0.005	2.760±0.007 1.456±0.003
AFECP(6.25)	2.950± 0.091 1.522± 0.053	27.131± 0.00 14.560± 0.030	1.620±0.008 0.857±0.005	1.170±0.010 0.618±0.005
%Inhibition/p value	62.710%/p<0.0001	19.780%/p<0.0001	10.035%/p<0.0001	57.554%/p<0.0001
Control	19.530± 0.249 10.280± 0.127	12.249± 0.639 8.440± 0.329	1.850±0.013 0.962±0.005	2.069±0.005 1.008±0.003
Albendazole	11.402±0.157 6.002± 0.083	8.130±0.336 6.295±0.169	1.340±0.015 0.705±0.008	0.727±0.006 0.383±0.001
%Inhibition/p value	41.617%/p<0.001	25.650%/p<0.005	26.611%/p<0.0001	64.797%/p<0.0001

Values are expressed as mean± S.D. Each test was done in triplicate. (p<0.0005 extremely significant, p<0.005 very significant).

(b)

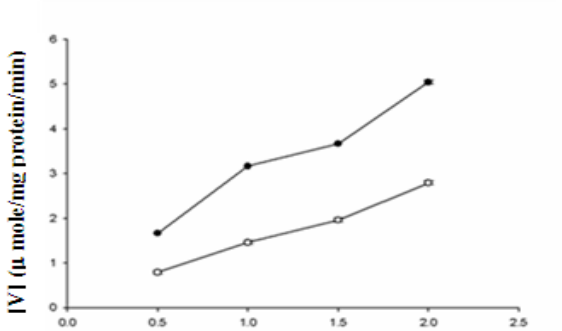


K_m(Control, EFECP) = 0.833

V_{max}(Control)=1.5

V_{max}(EFECP)=0.769

(a)



(a)

Figure.1. (a) Michaelis Menten graph sh effect of EFECP on specific activity $[V]$ of ALP taken in units/mg protein/min at different substrate concentration $[S]$ in µmoles in *G. indicus*

(b) Lineweaver Burk plot showing maximum apparent initial enzyme velocity in µ mole/mg protein/min (V_{max}) and substrate affinity constant in µM (K_m) of ALP in the presence of EFECP in *G.indicus*.

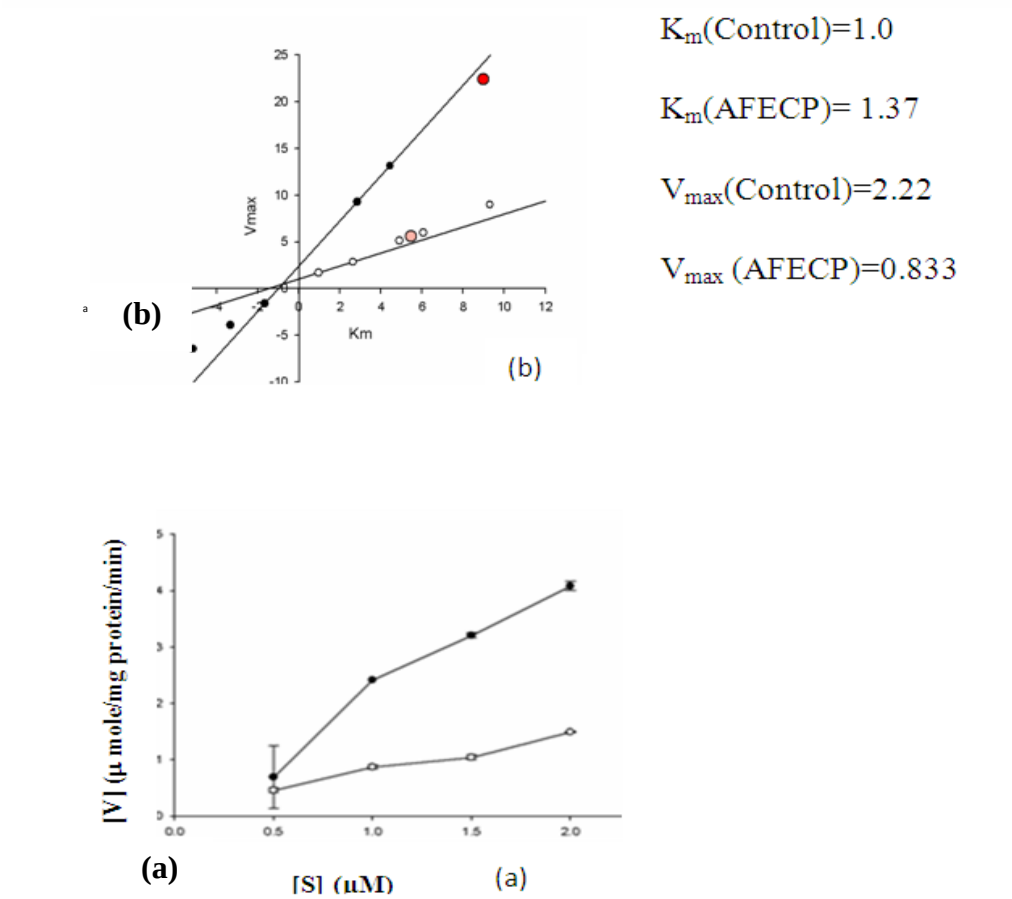
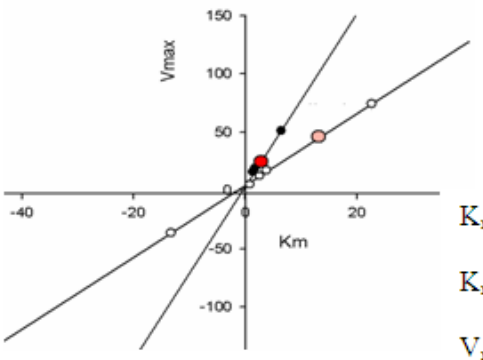


Figure.2. (a) Michaelis Menten graph showing effect of AFECP on specific activity $[V]$ of ALP taken in units/mg protein/min at different substrate concentration $[S]$ in µmoles in *G.indicus.*

(b) Lineweaver Burk plot showing maximum apparent initial enzyme velocity µ mole/mg protein/min (V_{max}) and substrate affinity constant in µM (K_m) of ALP in the presence of AFECP in *G.indicus*

(b)



K_m (Control) = 1.0

K_m (ABZ) = 2.1

V_{max} (Control, ABZ) = 5

(a)

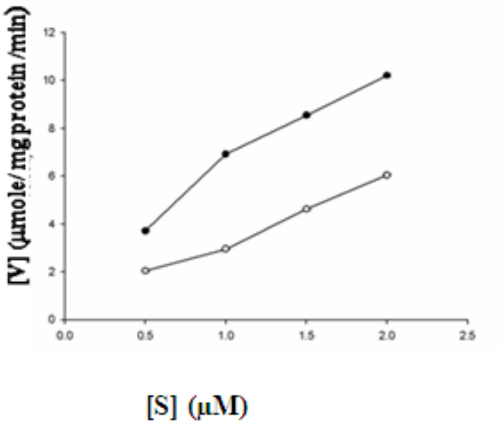
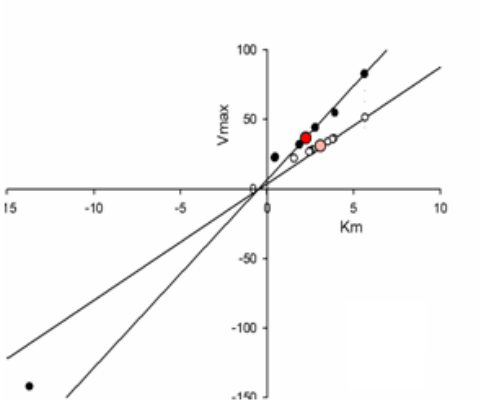


Figure.3. (a) Michaelis Menten graph showing effect of albendazole on specific activity [V] of ALP taken in units/mg/protein/min at different substrate concentrations [S] in µmoles in *G. indicus.*

(b) Lineweaver Burk plot showing maximum of apparent initial enzyme velocity in µ mole/ mg protein/ min (V_{max}) and substrate affinity constant in µM (K_m) of ALP in the presence of albendazole in *G. indicus*.

(b)



K_m (EFECP, Control)=0.769

V_{max} (Control) = 7.5

V_{max} (EFECP) = 5.0

(a)

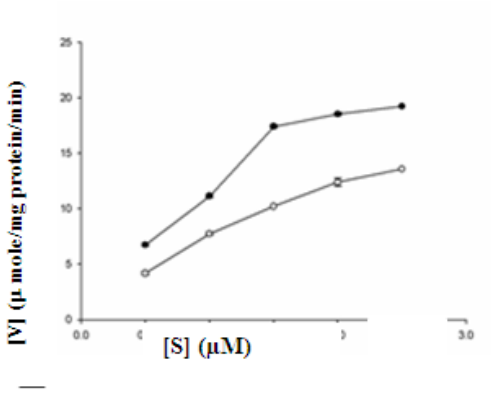
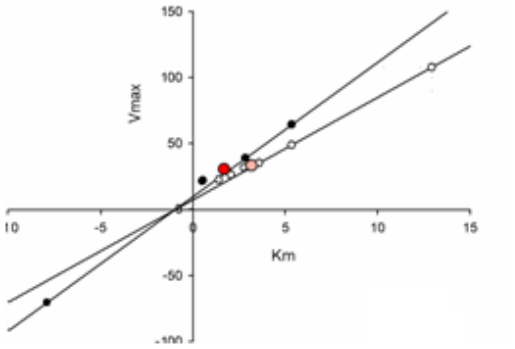


Figure.4. (a) Michaelis Menten graph showing effect of EFECP on specific activity $[V]$ of ACP taken in units/mg protein/min at different substrate concentration $[S]$ in µmoles in *G.indicus.*

(b) Lineweaver Burk plot showing max apparent initial enzyme velocity µ mole/mg protein/min (V_{max}) and substrate affinity constant in µM (K_m) of ACP in the presence of EFECP in *G.indicus.*

(b)



K_m (Control, AFECP) =1.17

V_{max} (Control) = 8.335

V_{max} (AFECP) = 6.25

(a)

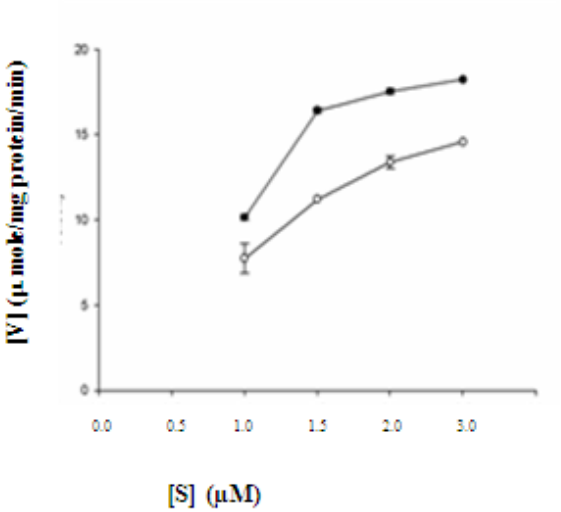
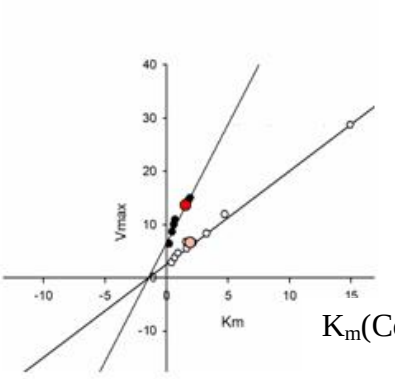


Figure.5. (a) Michaelis Menten graph showing effect of AFECP on specific activity $[V]$ of ACP taken in units/mg protein/min at different substrate concentration $[S]$ in µmoles in *G. indicus.*

(b) Lineweaver Burk plot showing max apparent initial enzyme velocity µ mole/ mg protein/min (V_{max}) and substrate affinity constant in µM (K_m) of ACP in the presence of AFECP in *G. indicus.*

(b)



K_m(Control, albendazole) = 2

V_{max}(Control) = 7.5

V_{max}(albendazole) =3.33

(a)

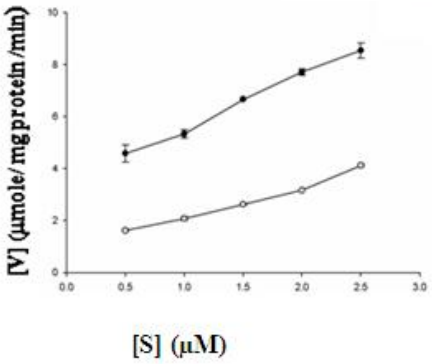


Figure.6. (a) Michaelis Menten graph showing effect of albendazole on specific activity [V] of ACP taken in units/mg protein/min at different substrate concentration [S] in µmoles in *G. indicus.*

(b) Lineweaver Burk plot showing maximum of apparent initial velocity in µ mole/ mg protein/min (V_{max}) and substrate affinity constant in µM (K_m) and of ACP in the presence of albendazole in *G. indicus.*