

Evaluation of anthelmintic activity of ethanolic and aqueous leaf extracts of *Azadirachta indica* on phosphatases in *Gastrothylax indicus*

Rama Aggarwal¹ and Upma Bagai²

¹(Parasitology Laboratory, Department of Zoology Panjab University, Chandigarh-160014,India.

²(Parasitology lab, Department of Zoology, Panjab University, Chandigarh-160014,India.

Abstract: Because of increasing anthelmintic resistance and the impact of conventional anthelmintics on the environment, it is important to look for alternative strategies against helminth parasite in sheep. The present study was performed to evaluate in vitro anthelmintic activity of plant *Azadirachta indica* on rumen amphistome of sheep. Anthelmintic activity of ethanolic and aqueous extracts of *A. indica* leaves in comparison with albendazole (positive control) and PBS (negative control) was evaluated through in vitro studies using worm motility inhibition and tegumental enzyme assays. *Gastrothylax indicus* was exposed in vitro to ethanolic and aqueous extracts (6.25 to 50 mg) of neem leaf to evaluate its anthelmintic efficacy. Mortality was observed at 2.14 ± 0.28 h and 1.71 ± 0.21 h for ethanolic and aqueous extracts respectively for the parasite at the highest test concentration of the plant extract. Anthelmintic albendazole was observed to cause mortality instantly (0.08 ± 0.01 h) at 80 µg/ml. Tegumental enzymes of the parasite ALP, ACP, ATPase and G-6-pase were found to be suppressed both by ethanolic and aqueous extracts. Enzyme kinetic studies showed inhibition to be non-competitive in case of ALP with both the extracts whereas for ACP it was found to be non-competitive with ethanolic and competitive with aqueous extract. Albendazole showed competitive inhibition in case of ALP and non-competitive with ACP.

Keywords: anthelmintic, *Azadirachta indica*, phosphatases, *Gastrothylax indicus*

I. Introduction

Major helminthic diseases of small ruminants (sheep/goat) include paramphistomiasis which cause considerable economic losses characterized by acute parasitic gastroenteritis, lowered meat and hair production leading to high mortality in host. Intensive researches on the biological evaluation of neem (*Azadirachta indica* A. Juss) products are being conducted in several countries. Neem oil is used as an antiseptic and parasiticide in various cutaneous infections like ring worm and scabies. Neem leaf juice is used for expelling worms and curing skin diseases [1].

Phosphatases are known to play a variety of important roles at the transporting surfaces in extracellular digestion and phosphorylation of nutrients transported, secreted and excreted. Due to their presence at the absorptive surfaces, phosphatases react with the substances in the external milieu [2]. 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 *A. indica* and albendazole on phosphatases was studied in *Gastrothylax indicus*, in order to get a better insight 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

Leaves of neem (*Azadirachta indica*), were collected from in and around Chandigarh. The plant material was identified in Department of Botany, Panjab University, Chandigarh with Voucher number- neem 5028

2.2 Preparation of extracts

Leaves of neem (*A. indica*), were washed thoroughly, shade dried and grounded by motor driven grinder into powder form. Both ethanolic and aqueous plant extracts were prepared [3]. Ethanolic leaf extract of *A. indica* (ELEAI), was exhaustively extracted by mixing 80 gm of powdered plant material and adding approximately 300ml of ethanol in a soxhlet apparatus. Aqueous extract (ALEAI) 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 ELEAI and ALEAI under in vitro conditions [3]. 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 positive and negative control respectively. There were three replicates for each treatment concentration. Immediately after the mortality, the parasites incubated in 12.5 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 0°C. The supernatant served as source of enzyme activity for different enzyme assays. Alkaline phosphatase (ALP) and acid phosphatase (ACP) activity was assayed [4]. Glucose-6-phosphatase (G-6-pase) and adenosine triphosphatase (ATPase) were estimated [5,6].

2.5 Specific activity and Enzyme kinetic studies

Specific activities of the enzymes were expressed as the units of enzymes activity per mg protein. Protein contents of different samples were estimated [7]. V_{max} (maximum of apparent initial enzyme velocity) and K_m (substrate affinity constant) of the ALP and ACP were determined by assaying the enzymes at different substrate concentrations [8]. Similarly, V_{max} and K_m of the enzymes were determined of the treated parasite with ELEAI and ALEAI as a whole and albendazole in vitro system.

2.6 Statistical analysis

Statistical analysis were carried out by employing Graph pad software 3 and data was expressed as mean \pm 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 1 shows the mortality of worms treated with various concentrations of ELEAI, ALEAI and albendazole. The controls survived for 9.2 ± 0.23 h. 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 V_{max} and K_m due to ELEAI, ALEAI and albendazole have been summarized in table 2 and figs.1-6. In vitro incubation of *G. indicus* with ELEAI, ALEAI and albendazole inhibited the activity of enzymes. Varying degrees of inhibition was observed with ELEAI, ALEAI and albendazole. ALP activity was inhibited 20.250% by ELEAI and 21.430% by ALEAI (Table2). V_{max} got lowered but K_m was not altered indicating the non- competitive behaviour of inhibition (Fig1,2). Inhibition was found to be statistically significant with both ELEAI and ALEAI (Table 2). With albendazole activity of ALP was reduced to 41.617% (Table 2). Inhibition was found to be competitive for ALP (Fig. 3) as K_m got increased in the presence of albendazole.

ACP activity was inhibited 5.092% by ELEAI and 11.825% by ALEAI (Table2). K_m was not changed but V_{max} was lowered with ELEAI showing non-competitive nature of the extract (Fig 4). But in case of ALEAI it was competitive as K_m was increased, making V_{max} same (Fig 5). Both ELEAI and ALEAI were found to be very significant for ACP. With albendazole Activity of ACP was reduced to 25.65% (Table 2).

Inhibition was found to be non-competitive in case of ACP as only V_{max} got lowered (Fig 6). Inhibition was found to be statistically extremely significant as compared to negative control (Table 2).

G-6-pase activity was reduced 17.670% by ELEAI but it was less than 10% with ALEAI. Albendazole showed the inhibition of 26.611%. ELEAI and ALEAI inhibited the activity of ATP ase nearly 30%. Albendazole showed the reduction of 64.797%. Inhibition was found to be extremely significant (Table2).

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 [9]. The results of the present study showed that *A.indica* leaf has good anthelmintic potential. The mortality of the worms (*G.indicus*) was much faster in albendazole treatment than in ELEAI and ALEAI. It is in agreement with the findings of earlier workers on different helminth parasites[3].

Tegumental enzymes play a very important role in maintaining the tissue homeostasis within the parasite. 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 [9].

In the present investigation, enzyme activities in ELEAI, ALEAI 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* [10]. Inhibition in the activity of G-6 Pase in *Trichuris globulosa* was shown with ethanolic neem leaf extract [11].

Enzyme kinetic studies showed inhibition to be non competitive in case of ALP with both ELEAI and ALEAI whereas albendazole depicted competitive kind. ACP was found to be non competitive with ELEAI and albendazole and competitive with ALEAI. 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 [11].

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 *A.indica* leaf 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.

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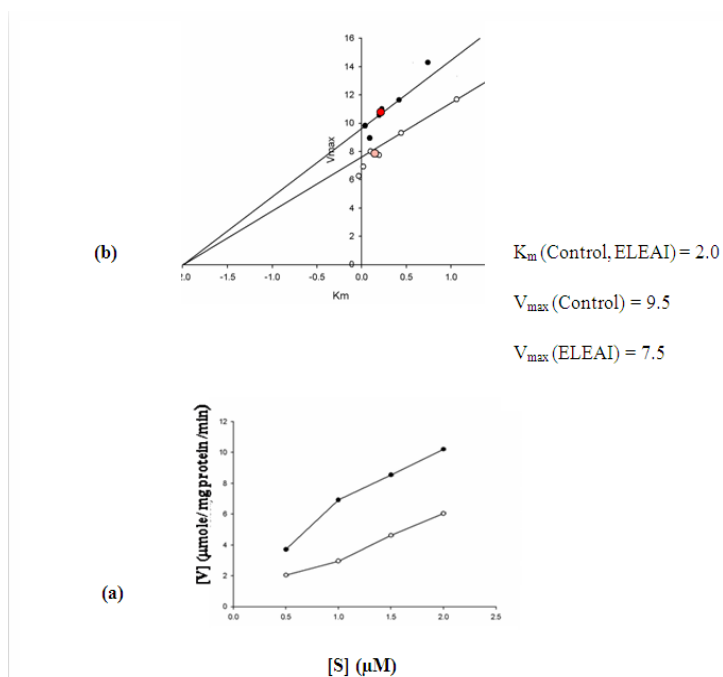


Fig 1

- Fig. 1 (a)** Michaelis Menten graph showing effect of ELEAI 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 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 ELEAI in *G. indicus*.

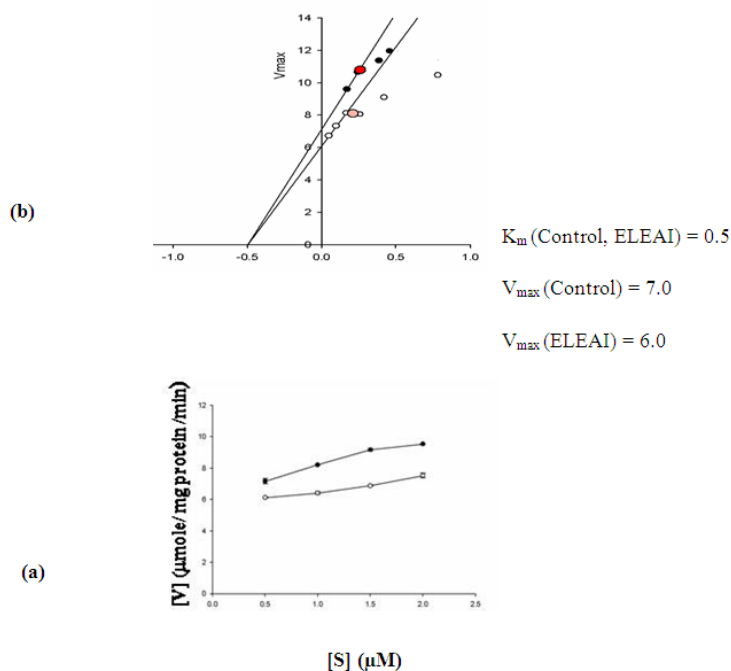


Fig 2

- Fig 2 (a)** Michaelis Menten graph showing effect of ALEAI 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 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 ALEAI in *G. indicus*.

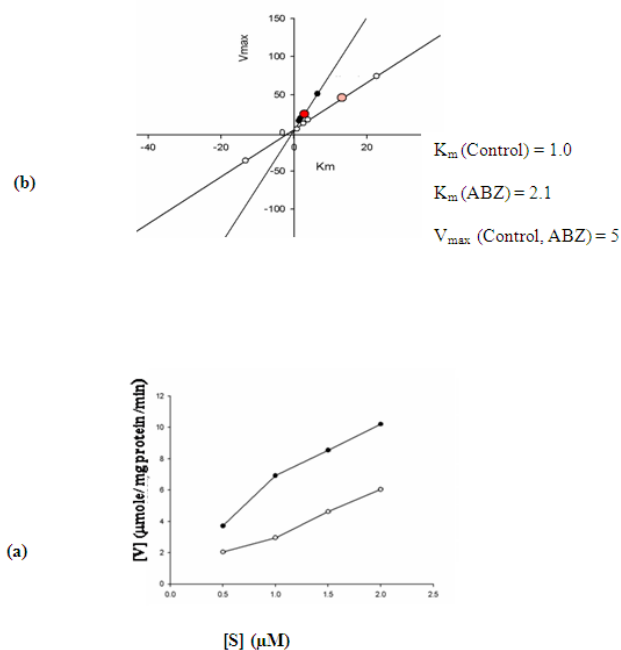


Fig 3

Fig 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 $\mu\text{ mole/ mg protein/ min}$ (V_{\max}) and substrate affinity constant in μM (K_m) of ALP in the presence of albendazole in *G. indicus*.

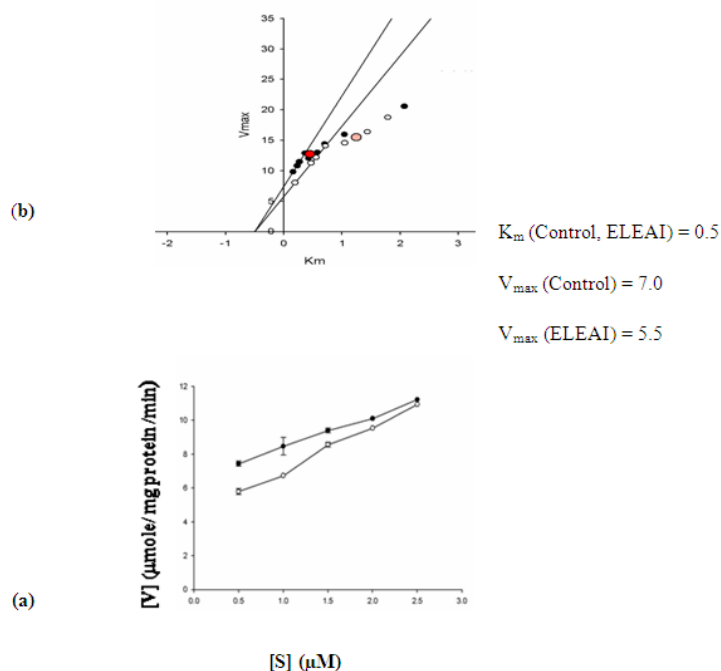


Fig 4

Fig 4 (a) Michaelis Menten graph showing effect of ELEAI 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 substrate affinity constant in μM (K_m) and maximum of apparent initial velocity in $\mu\text{ mole/ mg protein/ min}$ (V_{\max}) of ACP in the presence of ELEAI in *G. indicus*.

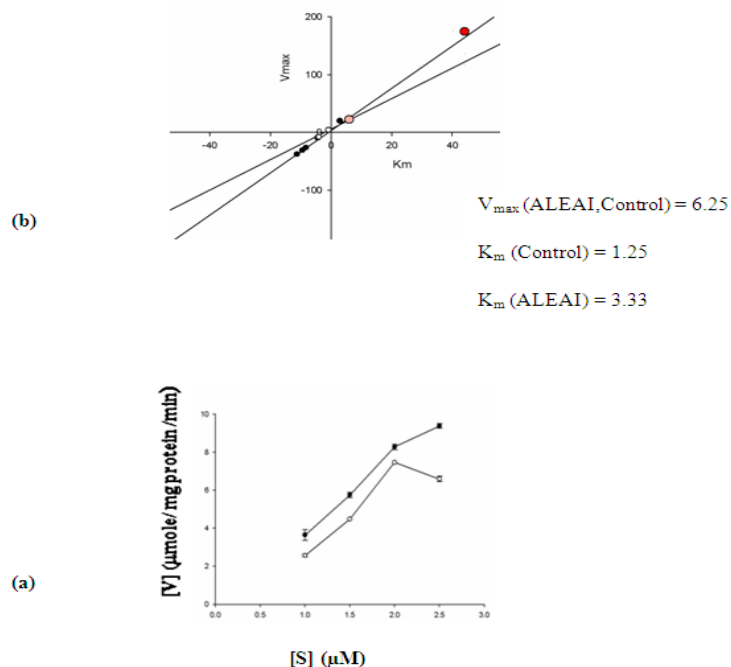


Fig 5

- Fig 5 (a)** Michaelis Menten graph showing effect of ALEAI 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 $\mu\text{ mole/ mg protein/ min}$ (V_{max}) and substrate affinity constant in μM (K_m) of ACP in the presence of ALEAI in *G. indicus*.

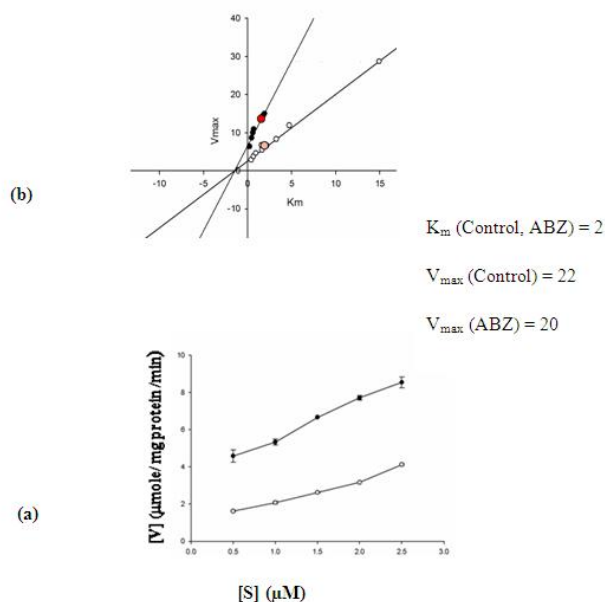


Fig 6

- Fig 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 $\mu\text{ mole/ mg protein/ min}$ (V_{max}) and substrate affinity constant in μM (K_m) and of ACP in the presence of albendazole in *G. indicus*.

Table 1. In vitro effect of plant extracts (ELEAI, ALEAI) and Albendazole on *G. indicus*

Dose Plant extract(mg/ml)/ Albendazole (µg/ml)	Time taken in hours for mortality of <i>G. indicus</i>			
	INCUBATION MEDIUM			
	ELEAI	ALEAI	ALBENDAZOLE	Control(PBS)
6.25/20	4.71±0.50	4.92 ± 0.50	3.25±0.12	9.2±0.23
12.5/40	4.21±0.42	4.28± 0.35	1.50±0.17	
25.0/60	3.14± 0.28	2.92± 0.28	0.50±0.02	
50.0/80	2.14± 0.28	1.71± 0.21	0.08±0.01	

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

Table 2. Effect of ELEAI, ALEAI 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	18.163± 0.091 9.459± 0.143	19.020± 0.300 10.010± 0.162	1.850±0.013 0.962±0.005	2.837±0.045 1.470±0.002
ELEAI(12.5)	14.460± 0.092 7.543±0.148	18.127± 0.162 9.500±0.030	1.524±0.015 0.792±0.008	1.868±0.014 0.982±0.006
% Inhibition/p value	20.250%/p<0.0001	5.092%/p=0.005	17.670%/p<0.0001	33.197%/p<0.0001
Control	18.163± 0.091 9.690± 0.108	15.815± 0.269 8.332± 0.183	1.850±0.013 0.962±0.005	1.056±0.061 0.545±0.016
ALEAI(12.5)	14.466± 0.091 7.613± 0.479	11.021± 0.300 7.347± 0.162	1.718±0.010 0.889±0.005	0.718±0.010 0.377±0.005
% Inhibition/p value	21.430%/p=0.001	11.825%/p=0.002	7.675%/p<0.0001	30.825%/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(20ug/ml)	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).