

1 RESEARCH ARTICLE

2 Regulatory role of Calcium Channel Blockers on
3 spontaneous muscular activity of *Gastrothylax*
4 *crumenifer*, a rumen amphistome

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10 ABSTRACT

11 Major proportion of intracellular calcium (Ca^{2+}) is achieved through opening of calcium channels present
12 in the plasma membrane which play an important role in regulating neuromuscular coordination and re-
13 lease of neurotransmitters from nerve terminals. Blockade of these calcium channels adversely affects
14 contractile process and release of neurotransmitters in majority of the neuromuscular preparations in vi-
15 tro. In present study, the cumulative addition of verapamil (10^{-7} - 10^{-3} M) caused marked excitation in am-
16 plitude, baseline tension and frequency of spontaneous muscular activity of *Gastrothylax crumenifer* a
17 rumen amphistome. Diltiazem (10^{-6} - 10^{-3} M) caused a significant and concentration-dependent increase in
18 amplitude and frequency of spontaneous muscular activity of isometrically mounted rumen amphistome. It
19 also caused significant rise in baseline tension at 10^{-5} to 10^{-3} M concentrations. Addition of nifedipine (10^{-7} -
20 10^{-3} M) elicited significant and concentration-dependent rise in amplitude and baseline tension, as com-
21 pared to control values without significantly effecting frequency of spontaneous contraction.

22 **Keywords:** comma separated keywords

23 *Gastrothylax crumenifer* is a common rumen am-
24 phistome belonging to class trematode. The infestation
25 of this parasite in ruminants results in decreased growth,
26 production and reproductive performance of productive
27 animals besides decreasing the quality and quantity of
28 animal products [1]. Synthetic anthelmintics currently
29 in use have long been considered the only effective way
30 of controlling these parasitic infections. Injudicious and
31 frequent use of these anthelmintics has resulted in the
32 development of resistance. Furthermore, residual toxic-
33 ity and adverse reactions in animals have been associ-
34 ated with the available synthetic anthelmintics [2,3].
35 Therefore, there is a need to develop specific drug(s)
36 targeting various macro-molecular components of these
37 parasites.

38 Neuromuscular system of helminthes is an important
39 area for target identification and drug development.
40 Acetylcholine, a major inhibitory neurotransmitter of
41 trematodes has been demonstrated immuno-
42 cytochemically to be present in peripheral and central
43 nervous system [4]. Calcium ions (Ca^{++}) play an impor-
44 tant role in neurotransmitter release from the nerve ter-
45 minals and neuromuscular coordination [5]. Bathing

46 medium free of Ca^{++} ions reduces the spontaneous mus-
47 cular activity of *Schistoma mansoni* [6]. Similarly in-
48 creasing external Ca^{++} ions concentration in the medium
49 mimics the inhibitory effect of ACh on spontaneous
50 muscular activity of split preparation of adult *Fasciola*
51 *hepatica* and *Hymenolepis diminuta* [7,8]. However,
52 calcium channels blockers, diltiazem and verapamil,
53 cause marked stimulation followed by paralysis of
54 *Schistoma mansoni* and *Fasciola gigantica*, respec-
55 tively. [9,10]. Contractions induced by calcium-
56 dependent depolarization have been observed in dis-
57 persed muscle fibres of *Schistoma mansoni* [11]
58 whereas nicardipine, a calcium channel blocker, blocks
59 these contractions [12].

60 Calcium currents have been recorded from muscle
61 fibers of *Bdelloura candida*. However, Ca^{++} currents
62 could not be recorded from muscle fibers of *S. mansoni*
63 [13] and *F. gigantica* [14]. Recently, a number of com-
64 ponents vital for Ca^{++} storage and release involving Ry-
65 anodine receptors (RyR) present in sarcoplasmic reticu-
66 lum have been demonstrated in the genera of *Schisto-*
67 *somes* [15,16].

Table 1. Effects of verapamil, diltiazem, nifedipine, and on amplitude (g) baseline tension (g) and frequency (per 5 min) of spontaneous muscular activity of *Gastrothylax crumenifer*

Observations	Concentrations					
	Control	10 ⁻⁷ M	10 ⁻⁶ M	10 ⁻⁵ M	10 ⁻⁴ M	10 ⁻³ M
Diltiazem						
Amplitude (g)	0.49±0.03	0.51±0.03	0.56±0.04**	0.61±0.06**	0.64±0.06**	0.68±0.05***
Baseline tension (g)	0.20±0.02	0.21±0.02	0.23±0.03	0.24±0.02**	0.27±0.03**	0.29±0.02***
Frequency/5min.	43±4.09	47.5±6.14	49.5±5.20*	50±5.42*	54±4.11*	55.5±5.85*
Verapamil						
Amplitude (g)	0.44±0.05	0.45±0.05	0.47±0.04	0.55±0.03	0.58±0.03*	0.75±0.08***
Baseline tension (g)	0.15±0.01	0.17±0.02	0.19±0.02*	0.22±0.02**	0.24±0.02***	0.27±0.03***
Frequency/5min.	46.5±3.94	48.5±5.41	49.5±3.18	50±2.65	51±5.38**	53.5±4.69*
Nifedipine						
Amplitude (g)	0.58±0.05	0.67±0.04*	0.73±0.08*	0.75±0.06***	0.77±0.09***	0.80±0.09***
Baseline tension (g)	0.22±0.02	0.24±0.02	0.25±0.02	0.26±0.03*	0.29±0.03**	0.30±0.03***
Frequency/5min.	54±4.39	58.5±3.54	61±5.19	60±4.53	57±3.47	55±4.44

*= $p<0.05$; **= $p<0.01$; ***= $p<0.001$; as compared to the controls

The present study was planned to investigate the role of spontaneous muscular contractions were utilized to evaluate the effect of different concentrations of different groups of voltage sensitive calcium channel blockers on spontaneous muscular activity of isometrically mounted parasitic rumen amphistome, *Gastrothylax crumenifer*.

MATERIAL AND METHODS

Collection of rumen amphistomes

Mature and healthy *Gastrothylax crumenifer* were collected from the rumen of freshly slaughtered goats at local abattoir in warm (38±1°C) Hank's Balanced Salt Solution (HBSS) in an insulated container and brought to the laboratory. They were kept in the BOD incubator at 38±1°C until further use. The amphistomes (*G. crumenifer*) were identified before experimentation.

Tissue preparation and mechanical recording

The spontaneously active whole mature amphistome was mounted isometrically in HBS solution at 38±1°C for 2 h. There was no significant difference in amplitude as per the method described for *Gigantocotyle explanatum* [17]. In short, the amphistome was mounted with the help of two fine hooks. One hook was inserted 1-2 mm caudal to anterior sucker and fixed to the tip of aeration tube and another hook was pierced through the surface of acetabulum and connected to the isometric force transducer. The spontaneous muscular activity of isometrically mounted amphistome was recorded in Chart Window 4 Software programme. (Powerlab, AD Instruments, Australia).

Experimental protocol

Graded molar concentrations (10⁻⁷- 10⁻³ M) of different groups of calcium channel blockers; verapamil (Phenylalkylamine derivative) (Sigma, USA), diltiazem (benzothiazepine derivative) (Sigma, USA) and nifedipine (dihydropyridine derivative) (Sigma, USA) were applied after equilibration of the fluke to examine their effects on spontaneous muscular activity of *G. crumenifer*.

Data collection and statistical analysis

Three attributes, viz., the amplitude (average of all peaks per five minutes), baseline tension (average of all minimum levels of contractions used for measuring amplitude) and frequency (total number of contractions in

1095 min) of spontaneous muscular contractions were utilized to evaluate the effect of different concentrations of different groups of calcium channel blockers. Experiment with each drug was repeated at least six times on fresh isometrically mounted *G. crumenifer*.

The results are presented as mean ± standard error of mean and paired 't' test was applied to measure the level of significance [18].

RESULTS

The isometrically mounted amphistomes exhibited rhythmic phasic contractile activity continuously for 20 hours. The mean amplitude, baseline tension and frequency of the rhythmicity recorded every 15 min after applying the tension of 200 mg, were 0.42 ± 0.03 g (n=6), 0.13 ± 0.01 g (n=6) and 45 ± 2.90 contractions/5 min time period (n=6), respectively. The isometrically mounted amphistomes exhibited apparently uniform pattern of spontaneous muscular activity for a period of 272 h. There was no significant difference in amplitude (0.38 ± 0.02 g; n=6), baseline tension (0.11 ± 0.02 g; n=6) and frequency (41 ± 3.44/ 5 min; n=6) of spontaneous contractions recorded 2 h after mounting as compared with those recorded 15 min after applying the tension to the amphistome. The representative recording is given in Fig. 1.

Effect of calcium channel blockers on spontaneous muscular activity of *G. Crumenifer*

Diltiazem, a benzothiazepine derivative causes a significant and concentration-dependent (10⁻⁶ to 10⁻³ M) increase in amplitude and frequency of spontaneous muscular activity as compared with control amplitude (0.49 ± 0.03 g) and frequency (43.0 ± 4.09/ 5 min). It also caused marked rise in baseline tension at 10⁻⁵ to 10⁻³ M concentrations in a concentration-dependent manner as shown in Table 1 and Fig 2a and 2b.

Effects of verapamil, a phenylalkylamine derivative on control amplitude, baseline tension and frequency of spontaneous contractions of isometrically mounted rumen fluke is shown in Table 1, and Fig 3a and 3b. Verapamil in cumulative concentrations at an increment of 1 log unit produced significant excitation in the amplitude (at 10⁻⁴ and 10⁻³ M), baseline tension (10⁻⁶ to 10⁻³ M) and frequency (at 10⁻⁴ and 10⁻³ M) of spontaneous muscular activity of the rumen fluke.

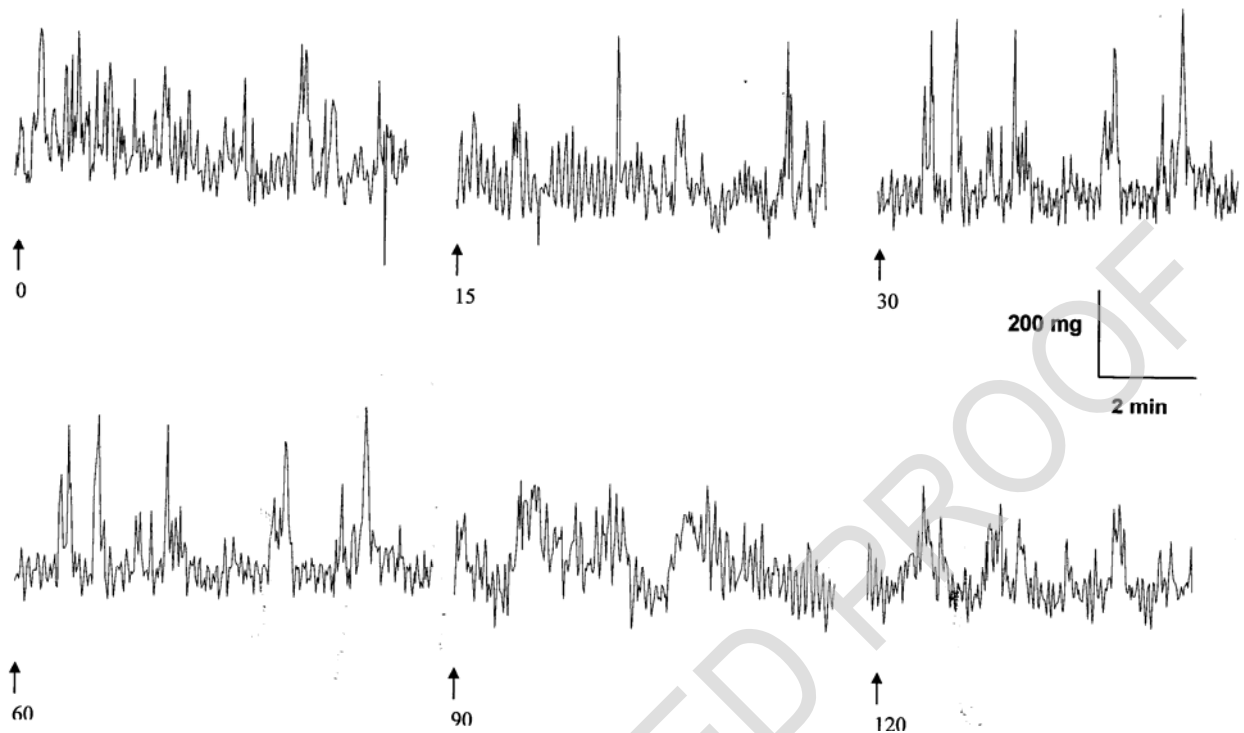


Fig. 1. Time-dependent control recording of spontaneous muscular activity of *G. crumenifer* (0 to 120 min)

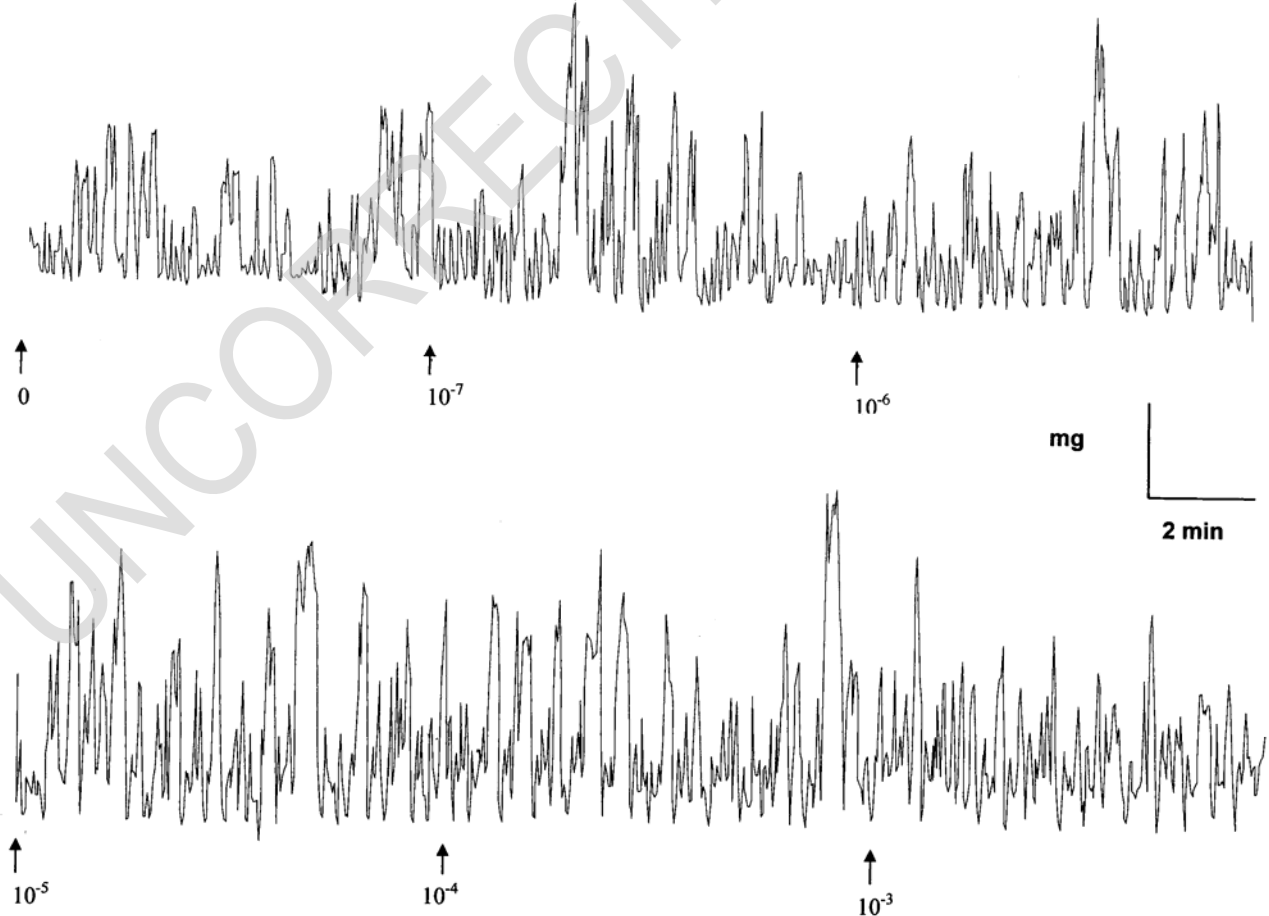


Fig. 2a. Effects of different concentrations (10^{-7} M to 10^{-3} M) of Diltiazem on spontaneous muscular activity of *G. crumenifer*

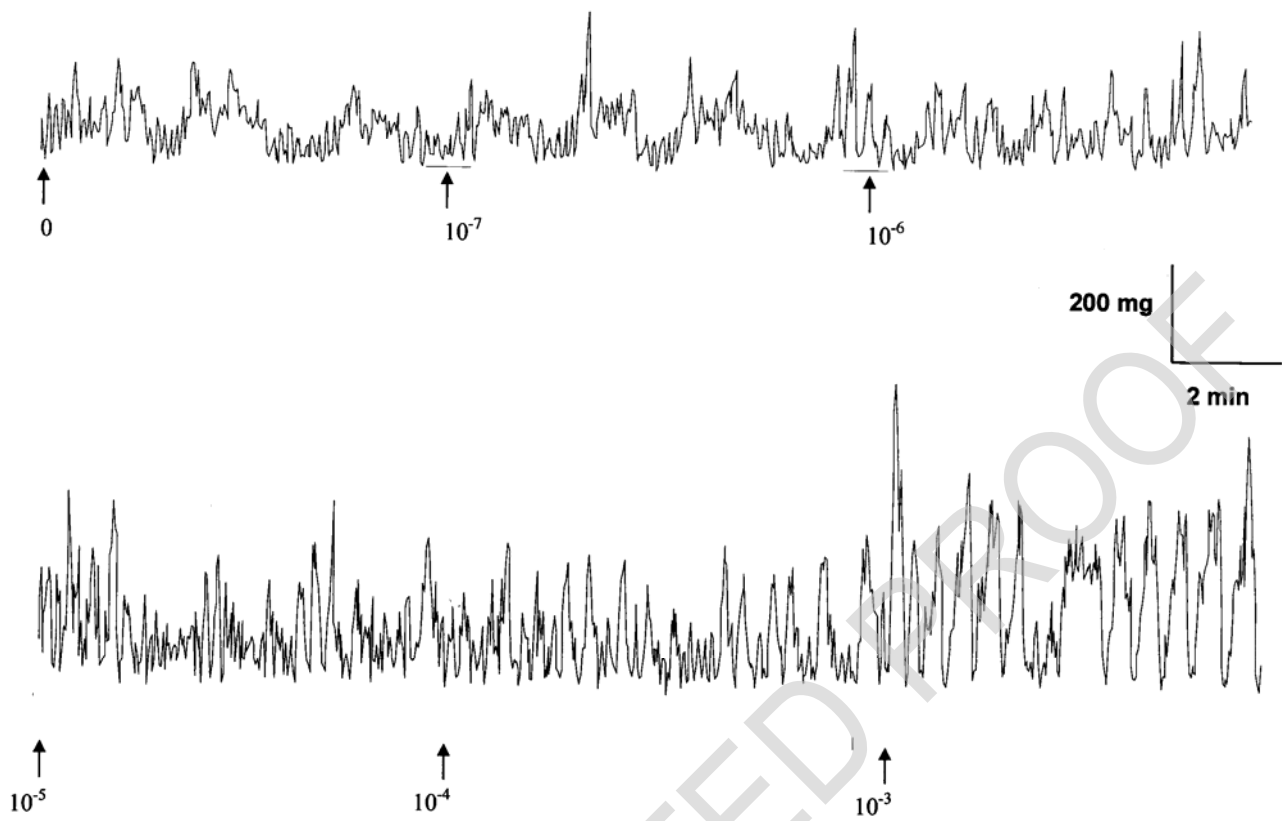


Fig. 3a. Effect of different concentrations (10^{-7} M to 10^{-3} M) of Verapamil on spontaneous muscular activity of *G. crumenifer*

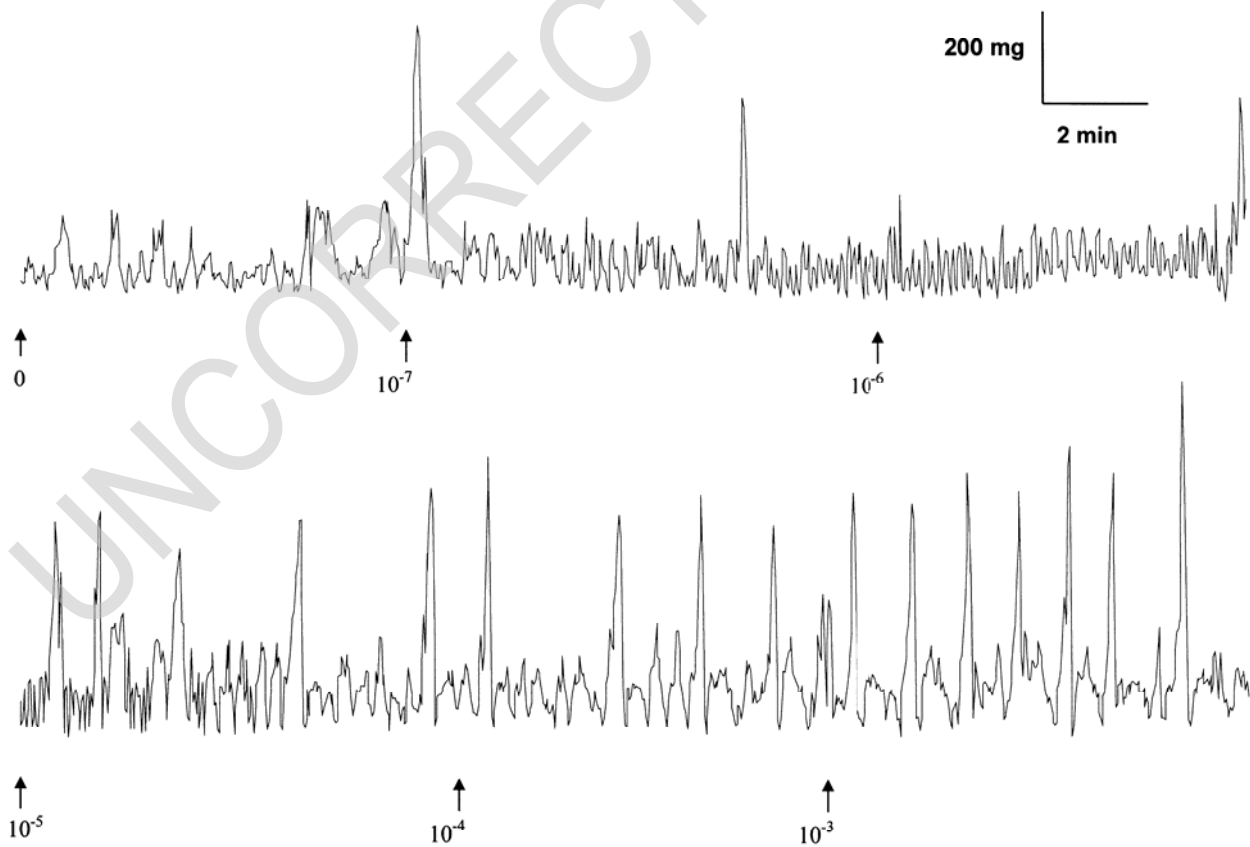


Fig. 4a. Effects of different concentrations (10^{-7} M to 10^{-3} M) of Nifedipine on spontaneous muscular activity of *G. crumenifer*

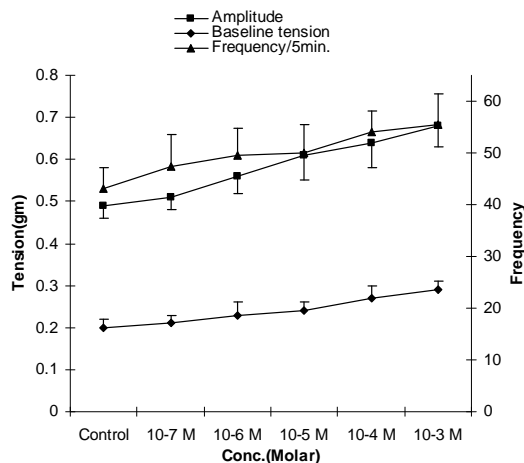


Fig. 2b. Effect of different concentrations (10^{-7} M to 10^{-3} M) of Diltiazem on amplitude (g) baseline tension (g) and frequency (per 5 min) of spontaneous muscular activity of *G. crumenifer*

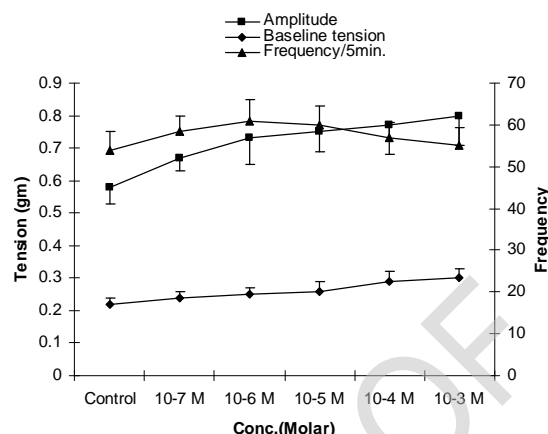


Fig. 4b. Effect of different concentrations (10^{-7} M to 10^{-3} M) of Nifedipine on amplitude (g) baseline tension (g) and frequency (per 5 min) of spontaneous muscular activity of *G. crumenifer*

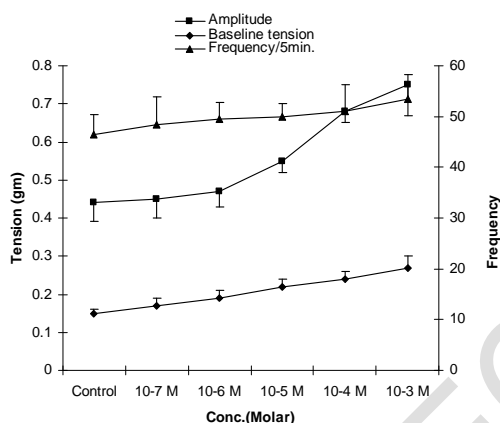


Fig. 3b. Effect of different concentrations (10^{-7} M to 10^{-3} M) of Verapamil on amplitude (g) baseline tension (g) and frequency (per 5 min) of spontaneous muscular activity of *G. crumenifer*

176 with a variation in the intensity. Out of these different
177 groups dihydropyridine derivatives are producing pro-
178 nounced contractile responses as compared to other
179 blockers. The results are in agreement with the earlier
180 reports on *S. mansoni* [9] and *F. gigantica* [10]. How-
181 ever, it has been shown that Ca²⁺-free bathing medium
182 reduced the spontaneous muscular activity of *S. man-*
183 *soni* [6], while increasing external calcium ion concen-
184 tration, mimicked the inhibitory effects of ACh on spon-
185 taneous muscular activity of split-preparation of adult *F.*
186 *hepatica* [7]. The probable mechanism may be that
187 these calcium channel blockers inhibit the release of
188 inhibitory neurotransmitters at the nerve terminals as it
189 is well documented that the release of neurotransmitter
190 at nerve terminal requires Ca²⁺ [5] or these calcium
191 channel blockers may be producing a direct stimulatory
192 effect on trematode neuromuscular system. Verapamil
193 and diltiazem have significant stimulatory effects on
194 muscular contraction whereas Nifedipine did not have
195 significant effect on the frequency of the activity. This
196 may be due to reversal of action of sodium-calcium ex-
197 changer proteins resulting from blocking of calcium
198 channels [19]. It is likely that similar mechanism operates
199 in muscular tissue of *G. crumenifer* and may be respon-
200 sible for Nifedipine not to significantly increase the fre-
201 quency of spontaneous muscular activity in *G. cru-*
202 *menifer*. It will be interesting to study the exact mecha-
203 nism for this excitatory effect on spontaneous muscular
204 activity with calcium channel blockers in trematodes at
205 molecular level.

DISCUSSION

164 Intracellular calcium (Ca²⁺) is responsible for the
165 muscular contraction and release of neurotransmitters
166 from nerve terminals in mammals. Removal of extra-
167 cellular Ca²⁺ and /or blockade of calcium channels ad-
168 versely affect contractile process and release of neuro-
169 transmitters in majority of the neuromuscular prepara-
170 tions *in vitro*. In the present study, however, calcium
171 channel blockers from different groups elicited an exci-
172 tatory response in amplitude and baseline tension of
173 muscular activity of *G. crumenifer*. The effect of the
174 calcium channel blockers on amplitude and baseline
175 tension in all groups show similar excitatory patterns

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155 Nifedipine, a dihydropyridine derivative at 10^{-7} to
156 10^{-3} M concentrations caused significant and concentra-
157 tion-dependent rise in amplitude compared with that of
158 control. At 10⁻⁵ to 10⁻³ M concentrations it also caused
159 concentration-dependent and significant increase in
160 baseline tension. Nifedipine did not show any concen-
161 tration-dependent and significant effect on frequency of
162 the fluke (Table 1 and fig 3a and 3b).

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