Annals of Phytomedicine 15(1): 846-855, 2026

DOI: http://dx.doi.org/10.54085/ap.2026.15.1.76

Annals of Phytomedicine: An International Journal
http://www.ukaazpublications.com/publications/index.php

Print ISSN : 2278-9839 Online ISSN : 2393-9885

Original Article : Open Access

In vitro biochemical and anthelmintic evaluation of ethanolic seed extract of *Butea frondosa* Roxb. Ex. Willd to establish mechanism of action through involvement of cholinergic pathway against *Paramphistomum cervi*

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Article Info

Article history
Received 9 January 2026
Revised 15 February 2026
Accepted 16 February 2026
Published Online 30 June 2026

Keywords
Paramphistomum cervi
Butea frondosa Roxb. Ex. Willd
In vitro biochemical estimation
Cholinergic pathway
Ion-channel

Abstract

The study aimed to establish the mechanism of action of ethanolic seed extract of *Butea frondosa* Roxb. Ex. Willd (ESEBF) as an anthelmintic against *Paramphistomum cervi*. ESEBF in various concentrations (100, 300, 1000, and 3000 µg/ml) was investigated to discover the mechanism of action on the spontaneous muscular activity (SMA) of the *P. cervi*. Different neurotransmitters belong to adrenergic, cholinergic, GABAergic, and serotonergic as well as channel modulators, namely; calcium, potassium, and NO channel were combined with various concentrations of ESEBF to observe their characteristics on SMA of *P. cervi*. When, administer ESEBF alone to the tissue bath to act upon the SMA of *P. cervi*, the concentration @ 3000 µg/ml was hyperpolarizing in nature and caused complete death of the worms. To explore the neurotransmitters involved for paralysis, it was found that there was no interaction of ESEBF with adrenergic, GABAergic, and serotonergic as well as calcium, potassium channels on SMA of *P. cervi* on combined experimentation. But, ESEBF @ 3000 µg/ml completely blocked the excitation of SMA produced by atropine (10-3 M) a cholinergic antagonist and observed similar type of behaviour with acetylcholine on SMA of *P. cervi*. Further, on combination of hemoglobin (10^{-3} M) and ESEBF @ 3000 µg/ml showed inhibitory response depicting antagonistic effect to excitatory response of hemoglobin indicating NO release for such relaxation.

1. Introduction

Commercial anthelmintics are the mainstay of the control of gastrointestinal helminth infections in a country like India. There are few things that makes livestock vulnerable to long-term and cumulative toxicity of synthetic anthelmintics. These include resistance to parasites and the presence of medication residues in milk, meat, wool, and most importantly, in pasture. In response to these circumstances, researchers have been working periodically to develop a plant-based anthelmintic that is resistant, less bio accumulative, and less toxic than synthetic anthelmintics. India is home to the medium-sized deciduous tree, Palash (*B. frondosa*), which is 10-15 m high and a member of the Fabaceae family (Saiyam *et al*., 2021). The chemical components of *B. frondosa* include alkaloids, as well as recently reported euphane triterpenoid esters and pterocarpans. The seed contains palasonin, d-methyl cantharidin, α-amyrin, β-sitosterol, alkaloid monospermine, glycerides of palmitic, stearic, linoceric, oleic, and linoleic acids, and proteolytic and lipolytic enzymes. *B. frondosa*, popularly referred to as 'flame of the forest,' has exhibited excellent anthelmintic properties, especially for roundworms (Singh *et al*., 2015). Also, the anthelmintic activity, toxicity, and other pharmacological properties of palasonin, the active principle of *B. frondosa* seeds and its piperazine salt has reported earlier (Raj and Kurup, 1968). The kymography tests with normally expelled human *Ascaris* showed that palasonin was better at killing *Ascaris lumbricoides* than either piperazine or santonin. The piperazine salt of palasonin was also much more effective than either palasonin or piperazine by itself.

The neurology of parasitic helminths is a field with very little knowledge. Pharmacological investigation is the only way to understand their differences from vertebrate receptors. The biochemical reason for the distinction between the contractile function of helminth muscle and vertebrate smooth and skeletal muscle is yet unknown. Therefore, the process of identifying an effective anthelmintic remains challenging and inefficient. Though, some studies explored the excitatory as well as inhibitory neurotransmitters on the spontaneous muscular activity of *P. cervi* but it is not enough to

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develop an anthelmintic based on those observations unless a solid pathway of mechanism of death of worms is discovered. The previous study on SMA of *P. cervi* revealed 5-HT, L-DOPA, and calcium chloride as excitatory while acetylcholine, GABA, glycine and potassium chloride as inhibitory neurotransmitters with reduction of amplitude, baseline tension and frequency of SMA (Barua *et al*., 2012; Saikia *et al*., 2013; Barua *et al*., 2014).

2. Materials and Methods

2.1 Ethical approval

The study was conducted after approval from the Institutional Animal Ethics Committee (IAEC), AAU, Khanapara, vide approval No. 770/GO/Re/S/03/CPCSEA/FVSc/ AAU/ IAEC/ 21-22/941 dated: 20.08.2022.

2.2 Collection of seeds of *Butea frondosa* Roxb. Ex. Willd and authentication

Butea frondosa Roxb. Ex. Willd seeds were obtained from Arunachal Pradesh and rural Assam. The seed material was authenticated by Dr. I. C. Barua, Principal Scientist from the Department of Agronomy, Assam Agricultural University, and the Voucher Specimen (6571, dated 14 September 2021) was preserved in their Institutional Herbarium and deposited in the Botanical Survey of India, Meghalaya, Shillong.

2.3 Collection of *P. cervi*

Mature and healthy *P. cervi* were collected from the rumen of freshly slaughtered cattle at a local abattoir in warm (37 ± 1°C) Hank's Balanced Salt Solution (HBSS) in an insulated container and brought to the laboratory. The worms were kept in the BOD incubator @ 37 ± 1°C until further use. The flukes were identified before experimentation using the procedure followed by Kumar (1999).

2.4 Chemicals

The majority of the chemicals utilized in the current study were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.5 Preparation of ethanolic seed extract of *B. frondosa*

250 g of powdered seed material was soaked in 1000 ml of ethanol for 72 h in a glass beaker. Every 24 h, the liquid was swirled with a sterile glass rod. After going through muslin fabric, the filtrate was obtained and concentrated @ 45-50°C under decreased pressure in a rotary evaporator (EQUITRON, Roteva, made by MEDICA INSTRUMENT MFG. CO. Mumbai-400013). Further the extract was dried over a water bath @ 37°C to achieve a semi-solid consistency. The extract was stored @ 4°C in an airtight container with appropriate labelling until its use.

2.6 Acute toxicity study

The guidelines of OECD-423 guided the performance of an acute oral toxicity study. The overnight fasted mice (n=3) were orally administered ESEBF at the limit dose of 2000 mg/kg body weight and observed continuously for behavioural, neurological, and autonomic profiles for 30 min, 1 h, 2 h, 4 h, 6 h, and then 24 h and 72 h and thereafter up to 14 days for any lethality, moribund state, or death.

2.7 *In vitro* motility study

Six adult *P. cervi* in each Petri dish having different dilutions of extract (ESEBF) in HBSS ranging from 50, 150, 300, 500, 1000, and 3000 µg/ml and standard oxyclozanide (10^{-5} M) were taken. However, the control Petri dish received only HBSS. The total volume of HBSS in each Petri dish was kept @ 5 ml. It was then incubated @ 37 ± 1°C for 5 h, and the number of live and dead adult worms was counted at 0, 30, 60, 90, 120, 150, 180, 210, 240, 270, and 300 min of exposure as described by Sujon *et al*. (2008).

% corrected mortality = Total mortality – mortality in the control group/ Total mortality

2.8 Study of biochemical parameters for evaluation of anthelmintic property of ESEBF and establishment of mechanism of action

To elucidate the possible mechanism of action of the plant extract, various biochemical parameters were studied. The effect of ESEBF in various concentrations (100, 300, 1000 and 3000 µg/ml) based on the *in vitro* motility study and standard reference drug oxyclozanide (10^{-5} M) (George, 2004) were studied. *P. cervi* (200-300 mg) were incubated in Petri dishes containing various concentrations of the plant extracts in 5 ml HBSS each for 5 h @ 37 ± 1°C in an incubator. After incubation, the worms or the incubate were used for biochemical studies.

2.8.1 Glucose uptake estimation

Glucose uptake study was conducted to detect the changes in glucose absorption by the rumen flukes in presence of plant extracts. These studies are based on the principle of Van den Bossche (1972). After 4 h of incubation the amount of glucose left in the incubate was estimated by the method described by Hultman (1959). Aldohexoses present in deproteinized sample react with O-toluidine reagent to give blue green colour of N-glycosylamine. The intensity of colour developed in the test sample is compared with that of standard glucose solution @ 630 nm (UV 2100, Chemito) and glucose content of the sample is computed.

2.8.2 Glycogen content estimation

After 4 h of incubation with extracts, the worms were washed 3 times with normal saline and their glycogen was precipitated and hydrolysed by method of Hassid and Abraham (1957). The hydrolysed glucose was estimated by the method described by Hultman (1959). The tissue containing glycogen was digested in hot concentrated potassium hydroxide, precipitated with ethanol and then hydrolysed with acid. On hydrolysis, 1 g glycogen gives 1.11 g of glucose.

2.8.3 Lactic acid estimation

After 4 h of incubation, the worms were rinsed thrice with normal saline and their lactic acid was estimated by the method of Gutmann and Wahlefeld (1974). The lactate in protein free filtrate is converted to pyruvate by lactate dehydrogenase in presence of NAD. NADH formed in the reaction is measured by the increase in extinction @ 340 nm (UV 2100, Chemito).

2.8.4 Acetylcholinesterase (AChE) estimation

The activity of acetylcholinesterase of whole worms was estimated by the method described by Fishman and Green (1961). This method

is based on the chemical determination of unreacted acetylcholine, which reacts with hydroxylamine to form acetyldroxamic acid, and further reacts with Fe^{+++} in acid solution to form a soluble red-purple colour complex, that can be estimated photometrically @ 540 nm.

2.9 Experimental procedures and mounting of *P. cervi* on isolated tissue bath

The adult *P. cervi* was positioned isometrically in HBSS solution @ 37 ± 1°C (Ahmed and Nizami, 1998). Two little hooks were used to help mount the worm. A tension of 200 mg was applied to the parasite using two hooks: one was inserted 12 mm caudal to the anterior sucker and fixed to the tip of the aeration tube; the other was pierced through the surface of the acetabulum and connected to the isometric force transducer (Powerlab, 4/35, 4Channel Data Acquisition System, Model: ML866/P, AD Instruments, Australia). Following equilibration, the tissue bath was supplemented with ESEBF @ different doses (100, 300, 1000, and 3000 µg/ml) in order to observe how they affect SMA of *P. cervi*. Three parameters such as amplitude, baseline tension, and frequency were measured for 10 min and recorded in the Chart Window 7 software programme. Before addition of different concentrations of ESEBF, control recordings were made for 15 min.

2.9.1 Evaluation of effect of ESEBF with the excitatory neurotransmitters and/or antagonist of inhibitory neurotransmitters on the SMA of *P. cervi*

ESEBF @ 3000 µg/ml was combined with different excitatory neurotransmitters as well as antagonists of inhibitory neurotransmitters in the tissue bath containing *Paramphistomum* to establish the interaction between the agents, and finally their effects on SMA of *P. cervi* were estimated.

2.10 Statistical analysis

The results are presented as mean ± standard error. Level of significance was analysed by using SPSS version 20.0 using one and two-way ANOVA.

3. Results

3.1 Percentage yield of extract obtained from seeds of *B. frondosa*

The percentage yield of the solid residue obtained during the extraction of ESEBF was found to be 14.35% w/w respectively.

3.2 Acute toxicity study

The study revealed no lethality or toxic manifestation after oral administration of ESEBF up to the limit dose, *i.e.,* 2000 mg/kg body weight, in mice. A similar result was reported by Devlekar *et al.* (2021), who found albino rats with a limit dose of *B. frondosa* hydroethanolic extract per os did not kill them or cause any other harmful effects.

3.3 Gross visual motility of *P. cervi*

Reduction of motility of parasite was evident as feeble at 5 h of exposure in presence of oxyclozanide (10^{-5} M). Similar type of result has also been observed with the ESEBF @ dose rate of 1000 and 3000 µg/ml (Table 1). From the *in vitro* motility experimentation, it has been noticed that worms @ 150 to 3000 µg/ml concentration of ESEBF caused death of parasite within 5 h of exposure and therefore a range of 4 concentrations were selected starting from 100 to 3000 µg/ml of ESEBF for further biochemical and neuromuscular study so that the reason of their death can be elucidated.

Table 1: *In vitro* gross motility of ESEBF against *P. cervi*

Drug/extract	Conc (µg/ml)	No of parasite showing motility (in min)										
		0	30	60	90	120	150	180	210	240	270	300
Control (HBSS)	-	6	6	6	6	6	6	6	6	6	6	6
ESEBF	50	6	6	6	6	6	6	5	5	4	3	2
	150	6	6	6	6	5	4	3	1	1	0	0
	300	6	6	5	4	2	1	0	0	0	0	0
	500	6	5	3	1	0	0	0	0	0	0	0
	1000	6	4	2	0	0	0	0	0	0	0	0
	3000	6	3	1	0	0	0	0	0	0	0	0
Oxyclozanide	10^{-5} M	6	2	0	0	0	0	0	0	0	0	0

3.4 Effect of ESEBF on the biochemical parameters (glucose uptake, glycogen content, lactic acid production and acetylcholinesterase activity) of *P. cervi in vitro*

There was significant ($p<0.05$) reduction of glucose uptake by ESEBF @ 1000 and 3000 µg/ml and reference drug oxyclozanide @ 10^{-5} M as compared to that of control (Figure 1). Similarly, glycogen content of *P. cervi* was reduced significantly ($p<0.05$) by ESEBF @ 1000 and 3000 µg/ml and oxyclozanide on comparing with the control (Figure 2). However, lactic acid production was increased significantly ($p<0.05$) by ESEBF @ 1000 and 3000 µg/ml and reference drug oxyclozanide @ 10^{-5} M as compared to the control (Figure 3). AChE activity, another biochemical estimation of *P. cervi* was significantly ($p<0.05$) inhibited by ESEBF @ 1000 and 3000 µg/ml and oxyclozanide @ 10^{-5} M as compared to control (Figure 4).

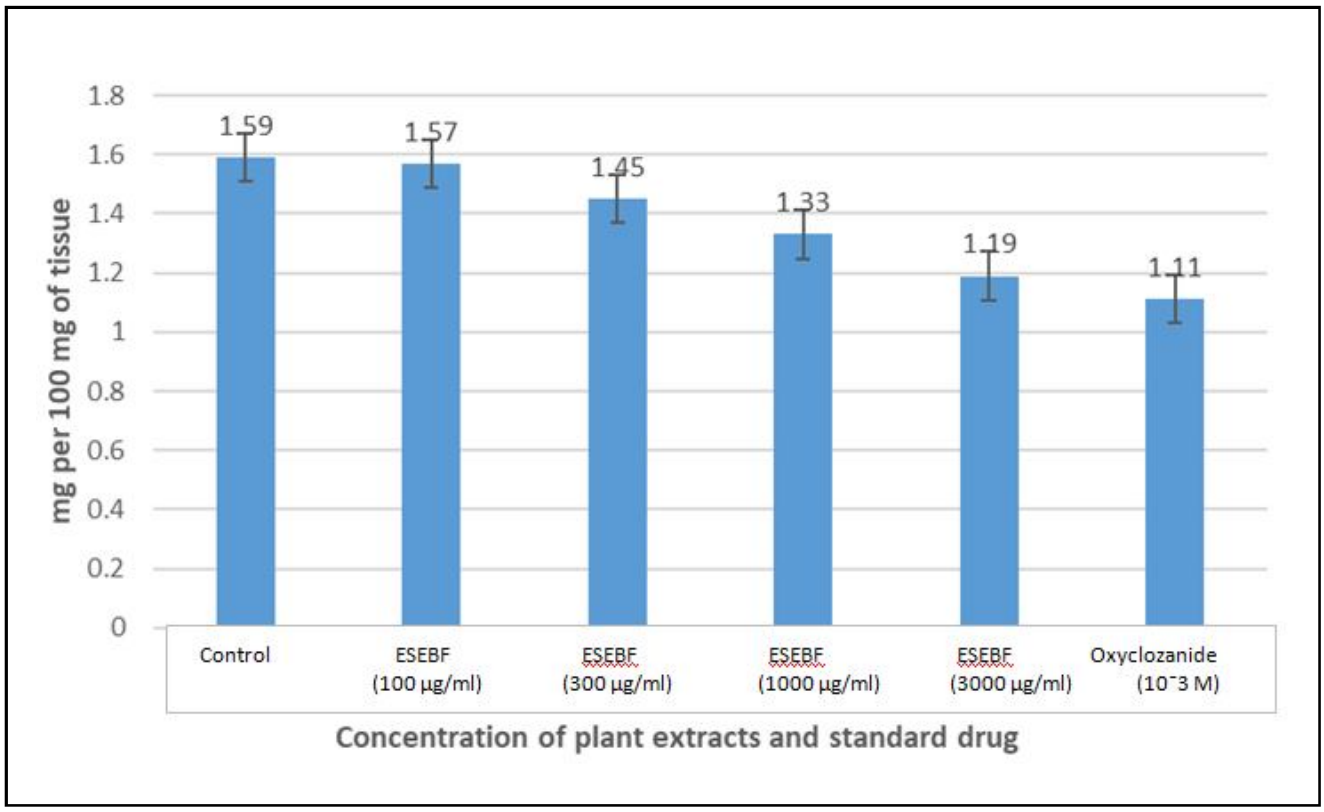

Figure 1: Effect of different concentration of ESEBF on glucose uptake following 4 h of incubation of adult *P. cervi* (*in vitro*).

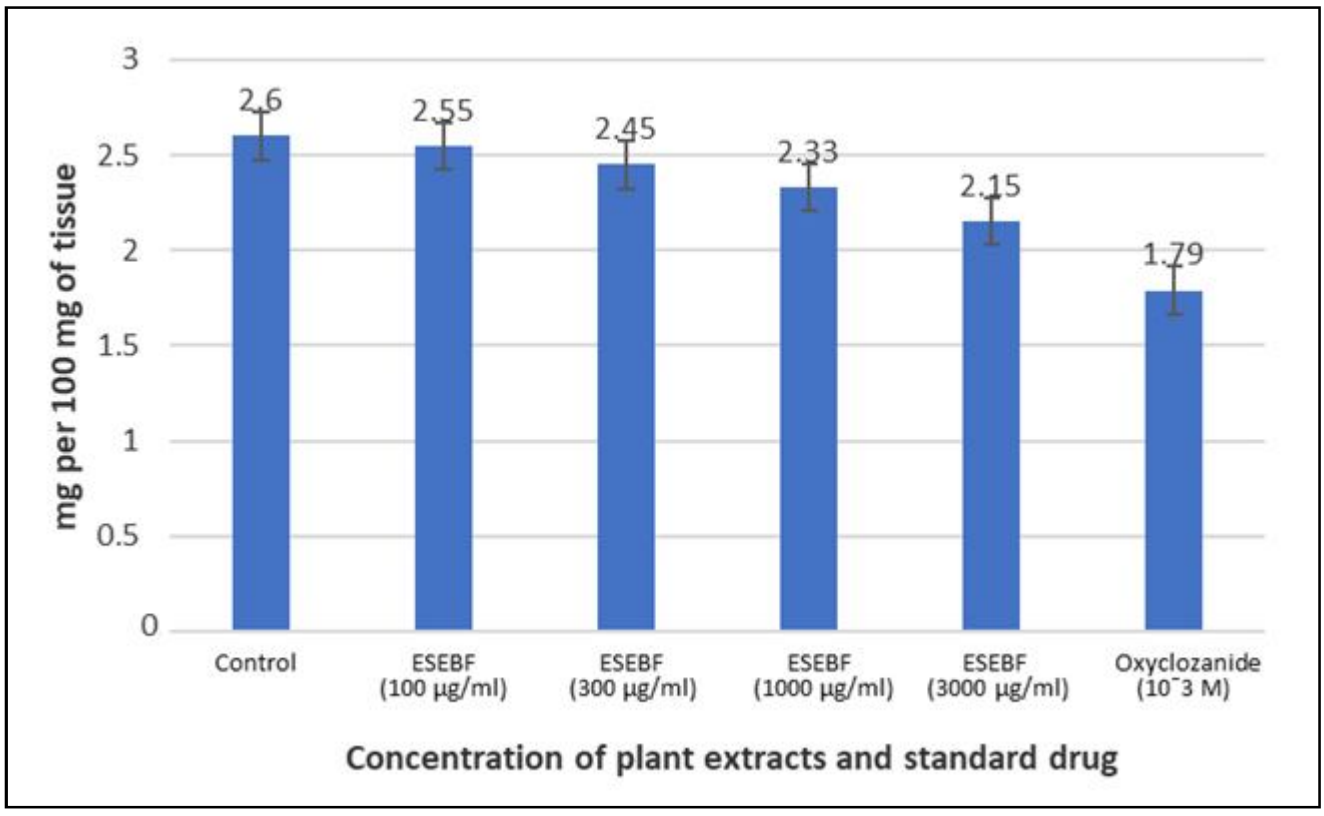

Figure 2: Effect of different concentration of ESEBF on glycogen content following 4 h of incubation of adult *P. cervi* (*in vitro*).

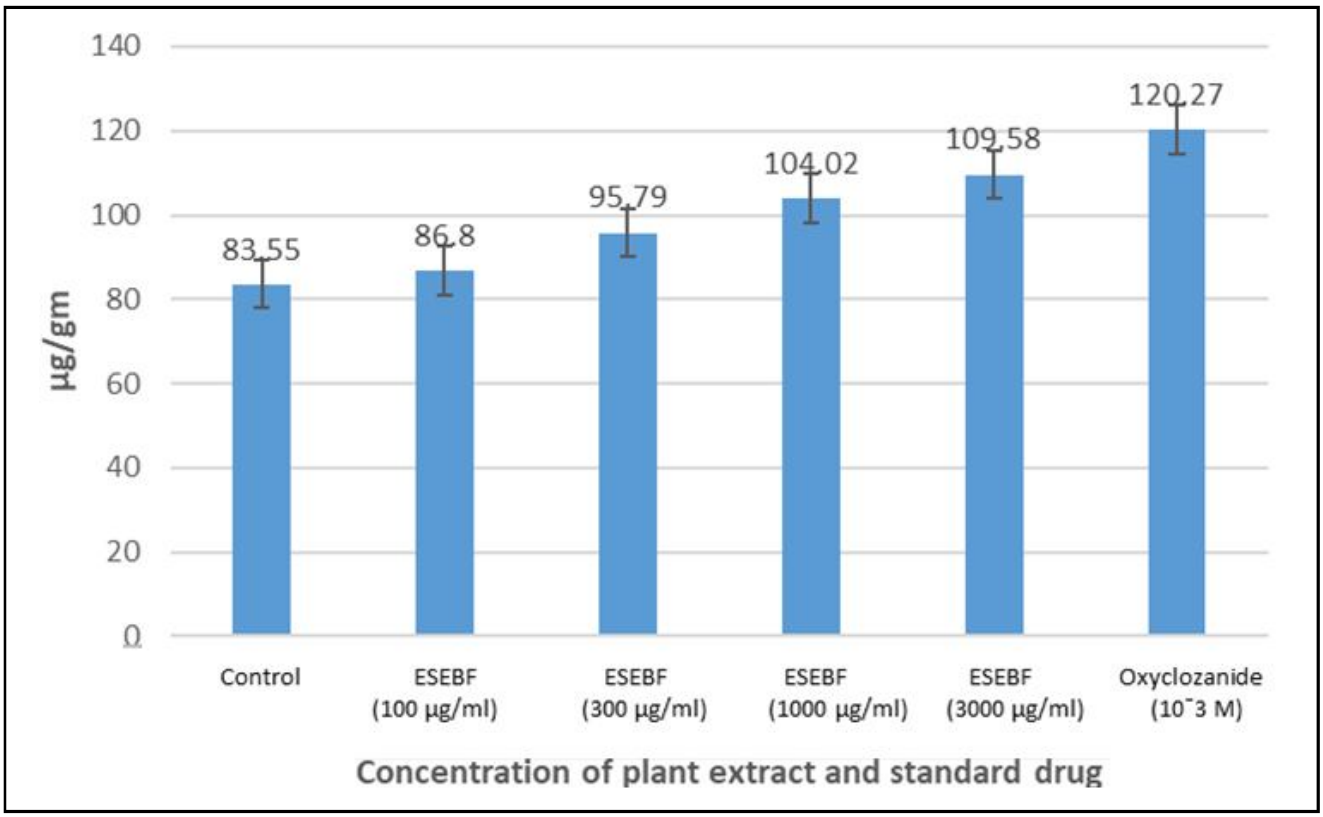

Figure 3: Effect of different concentration of ESEBF on lactic acid production following 4 h of incubation of adult *P. cervi* (*in vitro*).

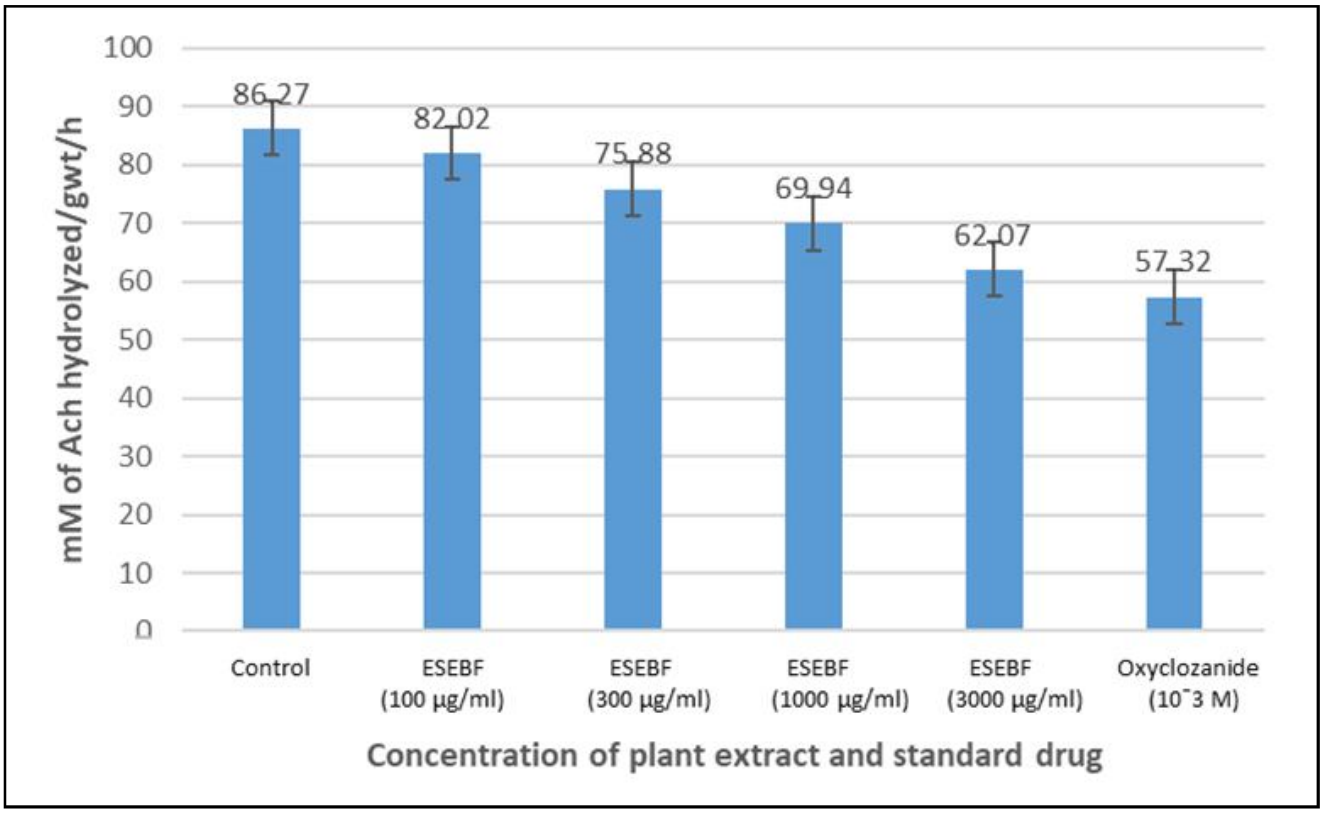

Figure 4: Effect of different concentration of ESEBF on AChE activity following 4 h of incubation of adult *P. cervi* (*in vitro*).

3.5 Effect of cumulative addition of ESEBF on SMA of *P. cervi*

In isometrically mounted mature *P. cervi*, the control (prior to application of drug) amplitude, baseline tension, and frequency of spontaneous muscular contractions were recorded to be 0.42 ± 0.06 g, 0.20 ± 0.03 g, and 53.72 ± 5.49 per 5 min, respectively. The ESEBF produced a hyperpolarizing effect on the rhythmic contraction of *P. cervi*. The amplitude of the *P. cervi* reduced significantly @ 300 µg/ml ($p<0.05$) and @ 1000 to 3000 µg/ml ($p<0.01$) concentrations. The baseline tension of the SMA was reduced significantly @ 1000 µg/ml ($p<0.05$) and @ 3000 µg/ml ($p<0.01$) concentrations as compared to the control value. Likewise, the frequency of the SMA was reduced significantly @ 300 µg/ml ($p<0.05$) and @ 1000 to 3000 µg/ml ($p<0.01$) concentrations as compared to control (Table 2). The inhibitory effect was maximum @ 3000 µg/ml of ESEBF, and the inhibition was such that the worm could not regain its normal contraction after constant washing (Figure 5).

Table 2: Effect of ESEBF on amplitude (g), baseline tension (g) and frequency (per 5 min) of SMA of *P. cervi*

Observations	Concentrations				
	Control	100 µg/ml	300 µg/ml	1000 µg/ml	3000 µg/ml
Amplitude (g)	0.42 ± 0.06	0.37 ± 0.06	0.32 ± 0.05*	0.26 ± 0.04**	0.20 ± 0.04**
Baseline tension (g)	0.20 ± 0.03	0.18 ± 0.02	0.15 ± 0.02	0.14 ± 0.03*	0.11 ± 0.03**
Frequency/5 min	53.72 ± 5.49	49.99 ± 5.14	43.65 ± 6.55*	37.06 ± 5.56*	31.55 ± 6.05**

Note: Values are the Mean ± S.E for six replicates. *$p<0.05$; **$p<0.01$ as compared to control.

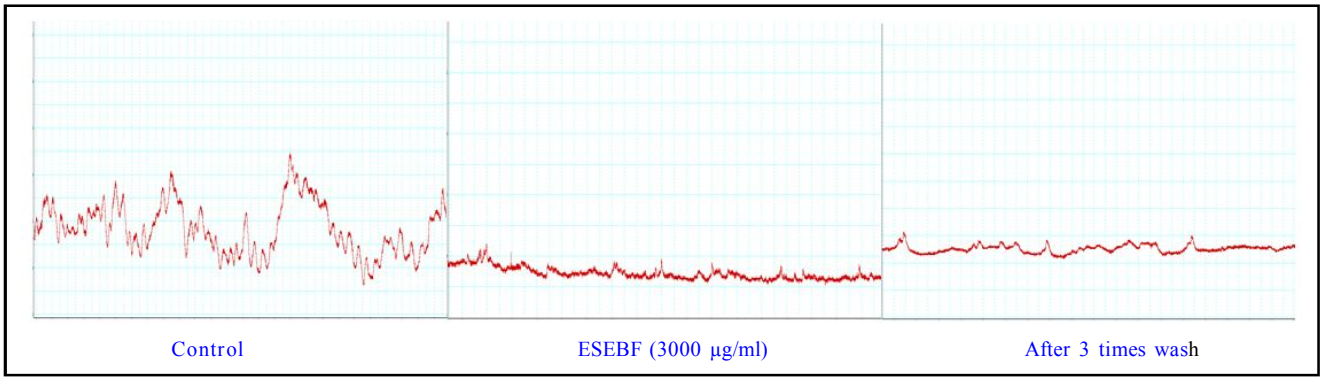

Figure 5: Effect of ESEBF (3000 µg/ml) on amplitude (g), baseline tension (g) and frequency (per 5 min) of SMA of *P. cervi*.

3.6 Effect of ESEBF with the excitatory neurotransmitters and/or antagonist of inhibitory neurotransmitters on the SMA of *P. cervi*

Upon assessing the individual effect of ESEBF on isometrically mounted *P. cervi*, it has been observed that the amplitude, baseline tension, and frequency of SMA, decrease concentration-dependently. Additionally, interaction of ESEBF was made with all the excitatory neurotransmitters (such as 5-HT, L-DOPA, and calcium chloride) and antagonists of inhibitory neurotransmitters (such as propranolol, picrotoxin, atropine, and 4-aminopyridine) to determine the likely mechanism behind the hyperpolarizing effect of ESEBF. It was found that ESEBF @ 3000 µg/ml could not able to antagonize the depolarization effect of SMA in *P. cervi* caused by excitatory neurotransmitters like 5-HT, L-DOPA, propranolol, picrotoxin, calcium chloride, and 4-aminopyridine. This meant that ESEBF @ 3000 µg/ml did not interact with these neurotransmitters (Table 3).

Table 3: Effect of ESEBF with the excitatory neurotransmitters (5-HT, L-DOPA, calcium chloride) and/or antagonist of inhibitory neurotransmitters (4-aminopyridine, picrotoxin, propranolol) on amplitude (g), baseline tension (g) and frequency of SMA of *P. cervi*

Drugs/agents	Parameters		
	Amplitude (g)	Baseline tension (g)	Frequency/5 min
Control	0.51 ± 0.07	0.19 ± 0.03	55.41 ± 5.82
5-HT (10^{-3} M)	0.78 ± 0.07*	0.27 ± 0.02*	74.37 ± 5.28*
5-HT (10^{-3} M) + ESEBF (3000 µg/ml)	0.79 ± 0.06^{NS}	0.28 ± 0.02^{NS}	75 ± 5.06^{NS}
Control	0.47 ± 0.05	0.22 ± 0.04	58.89 ± 6.58
Calcium chloride (10^{-3} M)	0.60 ± 0.06*	0.30 ± 0.03*	68.15 ± 5.66*
Calcium chloride (10^{-3} M) ESEBF (3000 µg/ml)	0.61 ± 0.06^{NS}	0.30 ± 0.02^{NS}	68.94 ± 5.74^{NS}
Control	0.55 ± 0.05	0.21 ± 0.04	57.03 ± 5.89

L-DOPA (10^{-3} M)	0.79 ± 0.06**	0.32 ± 0.03**	82.52 ± 5.79**
L-DOPA (10^{-3} M) + ESEBF (3000 µg/ml)	0.77 ± 0.05^{NS}	0.31 ± 0.02^{NS}	81.55 ± 5.15^{NS}
Control	0.46 ± 0.06	0.18 ± 0.04	51.28 ± 6.45
4-Aminopyridine (10^{-3} M)	0.68 ± 0.06**	0.29 ± 0.02**	69.41 ± 6.05*
4-Aminopyridine (10^{-3} M) + ESEBF (3000 µg/ml)	0.69 ± 0.06^{NS}	0.29 ± 0.03^{NS}	68.47 ± 5.28^{NS}
Control	0.52 ± 0.07	0.22 ± 0.03	58.74 ± 6.33
Picrotoxin (10^{-3} M)	0.74 ± 0.06**	0.31 ± 0.02*	72.52 ± 5.77**
Picrotoxin (10^{-3} M) + ESEBF (3000 µg/ml)	0.73 ± 0.06^{NS}	0.30 ± 0.03^{NS}	72.88 ± 6.21^{NS}
Control	0.41 ± 0.05	0.20 ± 0.04	48.72 ± 5.88
Propranolol (10^{-3} M)	0.58 ± 0.06**	0.27 ± 0.03*	62.80 ± 6.36*
Propranolol (10^{-3} M) + ESEBF (3000 µg/ml)	0.59 ± 0.05^{NS}	0.27 ± 0.02^{NS}	61.94 ± 5.75^{NS}

Note: Values are the Mean ± S.E for six replicates. *p<0.05; **p<0.01; NS (Non-significant) as compared to control.

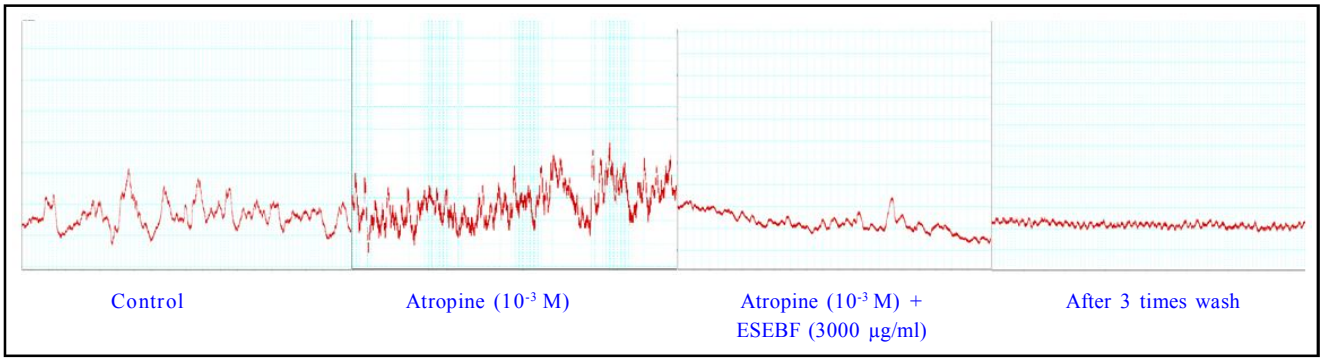

Figure 6: Combination effect of atropine (10^{-3} M) and ESEBF (3000 µg/ml) on SMA of *P. cervi*.

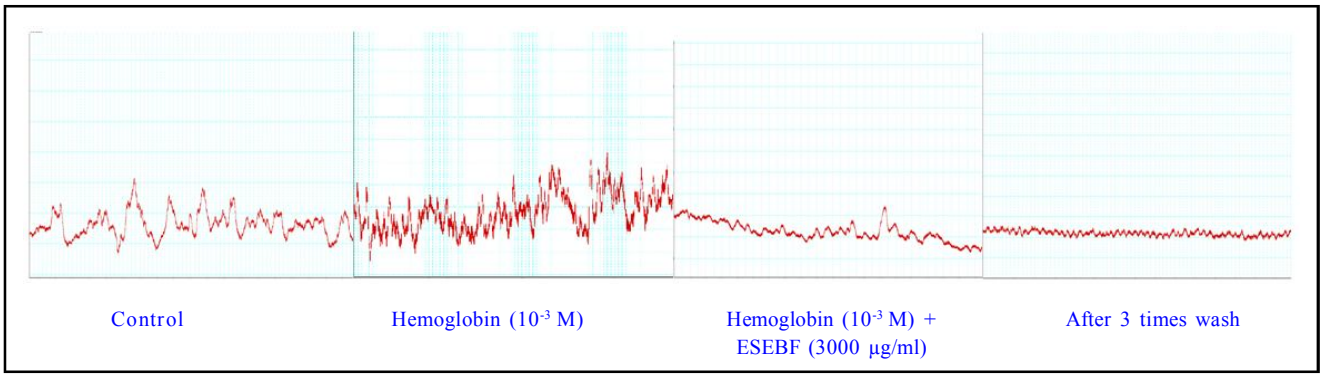

Figure 7: Combination effect of hemoglobin (10^{-3} M) and ESEBF (3000 µg/ml) on SMA of *P. cervi*.

Instead, when atropine @ 10^{-3} M was added to the tissue bath, the amplitude, baseline tension and frequency increased significantly (p<0.01). But on addition of ESEBF @ 3000 µg/ml to the tissue bath blocked the excitation on SMA produced by atropine and therefore, the amplitude, baseline tension and frequency were reduced significantly (p<0.01 and p<0.001) (Figure 6) indicating interaction of ESEBF with atropine @ 10^{-3} M.

Also, when hemoglobin @ 10^{-3} M was added to the tissue bath, the amplitude, baseline tension and frequency increased significantly (p<0.05). The amplitude, baseline tension and frequency were reduced significantly (p<0.01 and p<0.001) after addition of ESEBF @ 3000 µg/ml (Figure 7) indicating interaction of ESEBF with hemoglobin @ 10^{-3} M.

4. Discussion

A lot of selective anthelmintic drugs work on more than just the parasites' energy-generating system and reproduction (Geary *et al*., 1992). They also affect the parasites' neuromuscular system. Helminth parasites become paralyzed when exposed to certain drugs, such as pyrazine (Evans and Martin, 1996), levamisole (Harrow and Gration, 1985), piperazine (Martin, 1985), avermectin (Cully *et al*., 1994), anthelmintic organophosphates (Courtney and Robertson, 1995) and paziquantel (Mehlhorn *et al*., 1981).

To elucidate the possible mechanism of action of ESEBF various biochemical parameters, *viz*., glucose uptake, glycogen content and lactic acid production were taken as energy metabolism parameters.

Glucose is the only direct source of energy for parasitic trematodes (Tielens, 1997). Thus, hindrance in the uptake of glucose will affect the motility of the flukes. However, in the absence of readily available glucose, the parasite may mobilize its reserve glycogen to maintain its live support mechanism. Most of the anthelmintics act either by inhibiting glucose uptake and depleting the glycogen reserve of the parasite (Martin, 1997). Consequently, the energy production of the parasite is blocked. Decline in energy causes inhibition of motility of the flukes. Again, the elevated tissue lactic acid level implies that either malate pathway is inhibited or excretion of lactic acid from the parasite is blocked (Rahman and Bryant, 1977; Pampori *et al*., 1984). Hindrance in the malate pathway causes the inhibition of ATP production. So, the anaerobic glycolysis will be favored in which lactic acid is the end product. Thus, lactic acid is accumulated in the parasite which could be deleterious to the fluke. Motility of the parasite is declined due to lack of ATP.

Biochemical parameters of *P. cervi* were found to be altered in the presence of ESEBF. The energy metabolism of the worms seemed to be affected by ESEBF and it was observed by decline in the glucose uptake and glycogen content of *P. cervi* and increased lactic acid content at higher concentration.

Also, the present study discusses the effect of various concentrations of ESEBF on the SMA of *P. cervi in vitro*. The present study found that ESEBF significantly decreased the amplitude, baseline tension, and frequency of the SMA of *P. cervi* @ 300, 1000, and 3000 µg/ml. This observation was in agreement with the alcoholic extract of *M. philippinensis* in *F. gigantica* (Kusuwaha, 1998) and *G. crumenifer (*George, 2004). On considering the biochemical parameters and neuromuscular activity of *P. cervi* after application of different concentration of ESEBF, it can be explained that because of hyperpolarizing nature of ESEBF on SMA of *P. cervi*, it triggered the worms to get paralyzed and therefore, biochemical indicators chiefly glucose uptake and glycogen content got reduced significantly and accumulation of lactic acid got increased significantly at higher doses of ESEBF. All the consequences result in death of the worms within 1 h of exposure of ESEBF. Again, the AChE activity of *P. cervi* got inhibited significantly by higher doses of ESEBF signifying more concentration of ACh which ultimately affects the SMA of *P. cervi* leading to paralysis and death.

The nervous system of helminths is considered to consist of the neuronal cells and nerve junctions as well as the muscles they innervate. Drugs may act at any of a number of sites important in neuromuscular systems. These include the ionic mechanisms that help nerves send signals, the making, releasing, and breaking down of neurotransmitters and neurohormones (like neuropeptides), the receptors on nerves and muscles that pick up on these small molecules, and the second messenger system and ion channel that handle how the body reacts to new information. Tetra-hydro pyrimidine derivatives depolarize neuromuscular blocking agents in trematode parasites and the vertebrate host, acting on nicotinic acetylcholine receptors in nematode muscle cells. Reports indicate that they paralyze worms by causing a musculature contracture akin to acetylcholine action (Aubry *et al*., 1970). Levamisole exhibits a broad spectrum of antinematode activity across a variety of animal species. Levamisole has both muscarinic and nicotinic effects. These anthelmintics act by opening of non-selective cation ion channels that are permeable to both Na^+ and K^+ and leads to increase the membrane conductance and depolarize the membrane.

From the experiment it is confirmed that ESEBF have inhibitory response on SMA of *P. cervi* and the inhibition is such that @ 3000 µg/ml of ESEBF caused death of the *P. cervi*. Now to find out the particular system involved for such inhibition different excitatory neurotransmitters (Barua *et al*., 2012; Saikia *et al*., 2013; Barua *et al*., 2014) were combined with ESEBF @ 3000 µg/ml, so that the inhibitory effect of ESEBF can block the excitation produced by various excitatory neurotransmitters or antagonist of inhibitory neurotransmitters but after completion of the experiment it has been found that ESEBF @ 3000 µg/ml blocked the excitation on SMA produced by atropine revealing its own effect and therefore, it can be summarized that ESEBF behaves like cholinergic agonist on SMA of *P. cervi*. Further, on exploring the depth of its mechanism such as the ion channel involvement for hyperpolarizing action of ESEBF, various ion channel antagonists that are particularly excitatory (Barua *et al*., 2012; Saikia *et al*., 2013; Barua *et al*., 2014) in nature were combined with ESEBF. ESEBF @ 3000 µg/ml did not change the typical muscle contraction of calcium chloride, indicating that it does not function through the Ca^{2+} channel when combined with calcium chloride. Again, the SMA of *P. cervi* was unaffected by the combination of ESEBF @ 3000 µg/ml and 4-aminopyridine, an antagonist of the voltage-gated K^+ channel. This suggests that ESEBF also not act through the K^+ channel. However, when paired with ESEBF @ 3000 µg/ml and hemoglobin (NO scavenger), prevented typical excitatory response of hemoglobin @ 10^{-3} M, and an inhibitory response of ESEBF was visible in place of the excitatory response. Hemoglobin is a NO scavenger and therefore, NO concentration got reduced which is responsible for the excitation upon application of hemoglobin @ 10^{-3} M to the SMA of *P. cervi*. This excitation was blocked effectively by ESEBF @ 3000 µg/ml indicating involvement of NO signaling pathway. Based on this discovery, it can be deduced that ESEBF @ 3000 µg/ml may function *via* modifying the NO channel, resulting in *P. cervi* hyperpolarization and paralysis.

From the perusal of literature, it was evident that no work has been undertaken to establish the involvement of cholinergic neurohumoral transmission on NO pathway modulation in the parasites. From the above findings, it was observed that ESEBF behaved similar to that of ACh, as it interacts with atropine and blocks the excitatory response of atropine in *P. cervi*. Again, from the different channel interaction studies, it was found that ESEBF block NO scavenging and might activate NO release and cause paralysis of *P. cervi*. Now to validate these findings, Xu *et al*. (1996) demonstrated the relation between ACh and NO release from rat spinal cord. To validate above findings, much more research is needed, so that in near future an effective anti-trematodal drug of herbal origin can be developed.

5. Conclusion

The results of this study may help to incorporate *B. frondosa Roxb*. Ex. Willd in herbal remedies to treat *P. cervi*, particularly in Northeast

India. Additionally, by preventing the creation of resistance to synthetic anti-trematodal treatments, these findings may serve as a guide for the development of alternative anti-trematodal medications. As the experiment validate ESEBF is a strong contender of anthelmintic journey with establishment of mechanism of its action further additional *in vivo* study is required to confirm the validation of *in vitro* findings. Additionally, isolation and characterization of ESEBF is essential for further investigation with the isolated pure compound so that an effective herbal origin anti-trematodal anthelmintic can be developed in near future.

Acknowledgements

The authors are grateful to the Director of Research (Vety), Assam Agricultural University for providing physical facility to conduct this research.

Conflict of interest

The authors declare no conflicts of interest relevant to this article.

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Citation **S. Hazarika, S. Khargharia, H. Baruah, B. Borah, P. Konwar, D. Phangchupi, and C. Deori (2026). *In vitro* biochemical and anthelmintic evaluation of ethanolic seed extract of *Butea frondosa* Roxb. Ex. Willd to establish mechanism of action through involvement of cholinergic pathway against *Paramphistomum cervi*. Ann. Phytomed., 15(1):846-855, http://dx.doi.org/10.54085/ap.2026.15.1.76.**