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Original Article

Anthelmintic Activity of Fruit Extract and Fractions of *Piper longum* L. *In vitro*

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ABSTRACT
The present study was aimed to assess the *in vitro* ovicidal, larvicidal and adulticidal activity of methanolic extract and its fractions from fruits of *Piper longum* against strongyle ova, larvae and adult amphistomes respectively. The fruits of *P. longum* was identified and the accession number 006 was obtained. The phytochemical analysis revealed the presence of tannins, flavonoids, glycosides, phenolics, diterpenes and triterpenes in extract and fractions of *P. longum*. The extract and fractions were diluted serially in 6.25 per cent tween 80 to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 mg/mL. Ivermectin and thiabendazole at 10 µg/mL acted as positive controls and 6.25 per cent tween 80 as negative control. The methanolic extract was highly active against ova with IC_{50} of 0.026 mg/mL. The n-hexane fraction was potent in inducing larval mortality with IC_{50} of 1.383 mg/mL while chloroform fraction inhibited larval migration with IC_{50} of 1.796 mg/mL. Amphistomes were highly sensitive for methanolic extract of *P. longum* which possessed IC_{50} of 5.493 mg/mL Based on IC_{50} values, the methanolic extract was found to be most potent while chloroform fraction was effective against ova, larvae and also adults. GCMS analysis of potent methanolic extract revealed the presence of piperidinone, hydrocinnamic acid, ethylhexahydro azepine, methyleugenol, hexadecanoic acid and caryophyllene oxide which may have contributed for the anthelmintic activity. The acute oral toxicity study revealed mild vascular changes in liver. From the present study, it can be concluded that chloroform fraction of *P. longum* possessed maximum broad spectrum anthelmintic activity comparable to controls.
Key words: Adulticidal, GCMS, IC_{50}, Larvicidal, Ovicidal, *Piper longum*.

INTRODUCTION

Gastrointestinal nematode infections are one of the major causes for stunted economic development in livestock sector[1] due to loss of productivity among livestock in terms of milk, meat, wool, body weight gain etc.[1,2,3] Many of these helmintic infections have been reported to be zoonotic and thus have become a public health issue.[4] Though various methods like vaccination, biological control, resistant host etc. has been described, use of anthelmintic drugs (Benzimidazoles, Avermectins or Imidazothiazoles) have its own unique importance in combating these helminth infections.[5,6] Unfortunately, extensive and indiscriminate use and sole dependency on these chemical drugs resulted in occurrence of resistance to the drugs.[7,8] Failure to follow the specified withdrawal period after treatment led to accumulation of anthelmintic agent as residues in food chain.[9]

To control gastrointestinal nematode infections and to combat with rising anthelmintic resistance, it is very much necessary to think of some alternatives. This has highlighted the advantages of herbal remedies as an alternative. The plant derived natural substances have been used since past as anthelmintic agent and have been reported to be cheaper, more reliable and less toxic.[10] *Piper longum* (Tippali) belonging to the family piperaceae is widely used as medicinal plant for respiratory disorders, insomnia, epilepsy, hemiplegia, vitamin B_1 deficiency, fever, stroke and polypepsia.[11] In this study, the methanolic extract and its fractions were evaluated for ovicidal and larvicidal activity against strongyle ova and larvae respectively. The adulticidal activity was assessed against adult amphistomes.

MATERIALS AND METHODS

All the solvents and chemicals were of standard and analytic grade and the experimental solutions were freshly prepared.

Plant and extraction

The fruits of *Piper longum* were purchased from local vendors and was identified and authenticated at St. Thomas College, Thrissur, India. The voucher specimen was deposited at their repository and accession number was obtained. The collected fruits

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were shade dried, pulverized and subjected to methanolic extraction using soxhlet apparatus. The methanolic extract was dried using rotary vacuum evaporator (Bouchi) and further fractionated to obtain n-hexane, chloroform, n-butanol and aqueous fractions.[12] The extract and all fractions were stored under refrigeration (4°C) until further use.

Strongyle ova and larvae

The faecal samples were collected from various ruminant farms and were subjected to coprological examination. The ova were isolated by routine concentration method and the L_3 strongyle larvae were obtained by coproculture of positive faecal samples.[13,14]

Phytochemical screening of the extracts

The extract as well as the fractions was analysed qualitatively for various phytochemical constituents.[15]

In-vitro anthelmintic activity study

Extract dilutions and stock drug solutions

The methanolic extract and the fractions were serially diluted in 6.25 per cent tween 80 to obtain concentrations of 500, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 mg/mL. Ivermectin and thiabendazole at 10 µg/mL were used as positive control and 6.25 per cent tween 80 served as negative control.[16]

Egg hatch assay (EHA)

The egg hatch assay was done as per the standard procedures described previously.[17] Freshly isolated eggs were incubated in six well plates in serial dilutions of the plant extract/fractions of *P. longum,* as well as the standard drugs and solvent for 48 h, after which they were fixed with Lugol's iodine.[18] The numbers of hatched larvae and unhatched eggs were counted. The per cent inhibition of hatching and the concentration of extracts required to inhibit 50 per cent of the eggs from hatching (IC_{50}) were calculated.

Larval motility assay (LMA)

The larvicidal activity of methanolic extract and its fractions of *P. longum* were done according to previous publication.[19] The motility of third stage (L_3) larvae was recorded on exposure to the extract and fractions from fruits of *P. longum*. The cessation of motility of the larvae was considered as end of experiment. The motility of the larvae was observed by light microscopy at 6th and 12th h and the number of motile and non-motile larvae were counted. The results were expressed as per cent inhibition of larval motility.

Larval migration inhibition assay (LMIA)

The larval migration inhibition assay was done as described previously.[20] For LMIA, 1.8 mL of the test mixture containing 1690 µL water, 90 µL serially diluted concentrations of the extract of *P. longum*/ control drugs (10 µg/mL)/ 6.25 per cent tween 80 and 20 µL larval solution containing 90–100 larvae were added to each well of 24 well incubation plates. The plates were incubated in dark at 28°C for 24 h.

A second set of plates (migration plates) were prepared with 400 mL agar (bacto agar, concentration of 1.5%) in each well of row 1 and 3 of the 24 well plates. After 24 h, the suspensions from the incubation plates (liquid plus larvae) were carefully transferred onto the top of the sieves, suspended above the agar in rows A and C of the migration plate (Figure 1). Larvae were left to migrate for 24 h at 28°C. The ability of larvae to migrate was determined and expressed as per cent migration inhibition.

Adulticidal assay on amphistomes

Adulticidal activity was done as described earlier.[21] Extracts and fractions were diluted in tyrode's solution in petri plates to get a total volume of 20 mL. Tyrode's solution served as negative control while IVM and TBZ at 10 µg/mL each were used as positive control. Six amphistomes were placed in the extract/fractions containing petriplates and the motility/ wriggling movements of amphistomes were noted every 15 min and lack of movement even on stimulation indicated mortality of amphistomes. The time and concentration at which 50 per cent mortality was recorded.

Selection of potent extract/fraction

Quantification of dose required for 50 per cent anthelmintic response or the dose required to cause 50 per cent anthelmintic activity was calculated using computer software graph pad prism ver. 6 and is designated as inhibitory concentration-50 (IC_{50}). The potent extract/fraction was selected based on this IC_{50} values, obtained from the *in-vitro* studies.

FTIR and GCMS analysis of potent extract/fraction

Fourier transform infrared spectroscopy (FTIR)

FTIR spectrum of the potent extract/fraction was obtained by conventional KBr pellet/disc method using Perkin-Elmer FTIR spectrometer.[22] Two mg of extract/fraction and 298 mg of KBr (Potassium Bromide) was mixed and placed into an evacuable die on a hydraulic laboratory press and compressed under eight-ton pressure to form a pellet. The KBr pellet was placed in the pellet holder and spectrum was taken from 4000 cm^{-1} to 400 cm^{-1} wave number range using IR spectrophotometer against the blank (300 mg KBr pellet). The spectrum obtained was compared with FLUKA library provided by Perkin-Elmer.

Gas chromatography–Mass spectrometry (GC-MS)

The GC-MS analysis of the potent extract/fraction was carried out on Thermo Fisher Scientific TSQ 8000 GCMS, as per protocol described earlier.[23] The potent extract/fraction was dissolved in methanol, filtered in 0.22-micron syringe filter and the compounds were separated on TSQ-2MS capillary column (30 m × 0.25 mm; i.e., 0.25 µm film). The column oven temperature was programmed from an initial temperature of 40°C (3 min) to 160°C at 8°C min^{-1}, then to 300°C at 15°C min^{-1} with a final time of 10 min. The injection temperature and ion source temperature were 250 and 23°C, respectively. Helium was used as the carrier gas with a flow rate of one mL min^{-1}. The ionizing energy was 70 eV. All data were obtained by collecting the full-scan mass spectra within the scan range 40–350 amu. Compounds were identified using the National Institute of Standards and Technology (NIST) MS Search 2.0 library.

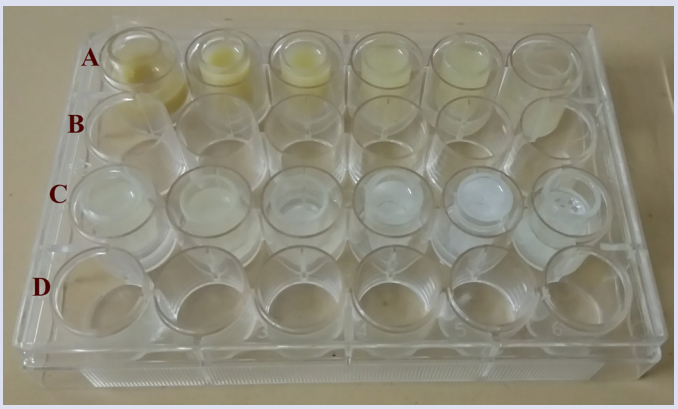

Figure 1: LMIA set up in a 24 well migration plate.

Acute toxicity study of potent extract/fraction

Acute oral toxicity study of the potent extract/fraction from fruits of *P. longum* was performed as per the Organization for Economic Co-operation and Development (OECD) guidelines for testing of chemicals, Test No. 420, acute oral toxicity-fixed dose (2000 mg/kg)[24]

Statistical analysis

The statistical analysis was performed for the data obtained. The IC_{50} values were computed using computer software Graph Pad Prism version 6.0. One-way ANOVA was employed to statistically analyse the data of *in vitro studies* of EHA, LMT and LMIA at P<0.05 using computer software SPSS version 21.

RESULTS

Phytochemical screening of the extracts

The methanolic extract and its fractions from fruits of *P. longum* were subjected to qualitative analysis of phytochemical constituents, the results of which are given in Table 1.

In-vitro anthelmintic activity study

Egg hatch assay (EHA)

The results of EHA for the extracts and fractions from fruits of *P. longum* are presented in Table 2. The efficacy of methanolic crude extract of *P. longum* in inhibiting hatching of strongyle eggs was found to be highest compared to all its fractions. Complete inhibition of egg hatching was observed at 500, 250 and 125 mg/mL which were more than inhibition produced by ivermectin and thiabendazole (98.97 and 99.26 per cent respectively) at 10 µg/mL. The methanolic extract, n-hexane and chloroform fractions of *P. longum* had IC_{50} of 0.026, 0.521 and 1.554 mg/mL respectively.

Larval motility assay (LMA)

The loss of complete motility caused by the methanolic extract and its fractions at sixth and 12th h is tabulated as per cent mortality in Table 3.

The methanolic extract of *P. longum* caused larval mortality of 100 per cent at higher doses of 125, 250, 500 mg/mL. The chloroform fraction of the methanolic fruit extract of *P. longum* was effective even at 1.95 mg/mL causing 52.31 per cent mortality of L_3 larvae and higher concentrations of 250 and 500 mg/mL produced mortality of 93.75, 97.33, 100 and 100 per cent respectively.

No much difference in mortality was observed at 12th h between the extract and fractions. The IC_{50} values was calculated using mortality data at 12th h. The methanolic extract, n-hexane and chloroform fractions of *P. longum* had IC_{50} of 1.533, 1.383 and 2.324 mg/mL respectively.

Larval migration inhibition assay (LMIA)

The results of LMIA for the methanolic extract and its fractions from fruits of *P. longum* are tabulated in Table 4. A dose dependent increase in activity was noted with the extracts. Larval migration inhibition of 89.66 per cent was observed at lowest dilution (500 mg/mL) of the methanolic fruit extract. An inhibition of larval migration by 92.73 per cent was noticed for highest concentration (500 mg/mL) of n-hexane fraction while the lowest concentration of fraction efficiently inhibited 44.23 per cent larvae from migrating.

The IC_{50} values for the methanolic extract, n-hexane and chloroform fractions of *P. longum* had IC_{50} value of 4.412, 2.301 and 1.796 mg/mL respectively.

Adulticidal assay on amphistomes

The lowest concentration and the time taken to induce 50 and 100 per cent mortality are given in Table 5. The methanolic extract of *P. longum* was highly effective produced 50 and 100 per cent mortality of adult amphistomes at concentration of 1.95 and 7.91 mg/mL respectively within 105and 85 min respectively. IC_{50} values for methanolic extract of *P. longum* was 5.493 mg/mL followed by n-hexane and chloroform fractions with IC_{50} of 105.1 and 21.91 respectively.

Selection of potent extract/fraction

Based on the IC_{50} values, the potent extract/fraction was selected Table 6. The IC_{50} of 0.026 mg/mL was obtained for methanolic extract of fruits of *P. longum* in egg hatch assay (EHA) which was lowest value in the

Table 1: Qualitative phytochemical analysis of extracts and fractions of *Piper longum*.

Phyto-constituents	Methanolic extract	n-hexane Fraction	Chloroform Fraction
Steroids	+	+	-
Alkaloids	+	-	+
Tannins	+	+	+
Flavonoids	+	+	+
Glycosides	+	+	+
Cardiac Glycosides	+	+	+
Phenolics compounds	+	+	+
Diterpenes	+	+	+
Triterpenes	+	+	-
Saponins	-	-	-

Table 2: Effect of methanolic extract and fractions of *Piper longum* on egg hatch, (% inhibition).

Concentration (mg/mL)	Treatments		
	MPL	n-HPL	CPL
1.95	88.89^{a}	75^{a}	58.33^{a}
3.91	91.94^{b}	87.27^{b}	70^{b}
7.81	89.09^{a}	88.46^{b}	80^{c}
15.63	94.12^{b,c}	93.88^{c}	94.55^{d}
31.25	97.78^{d,e}	100^{d}	100^{e}
62.5	96^{c,d}	100^{d}	100^{e}
125	100^{e}	96.67^{e}	100^{e}
250	100^{e}	97.5^{e}	100^{e}
500	100^{e}	100^{d}	100^{e}

Control	
6.25 % Tween 80	0
Ivermectin (10 µg/mL)	98.97
Thiabendazole (10 µg/mL)	99.26

Pooled SE: 1.191

Note: MPL: Methanolic *P. longum*; n-HPL: n-Hexane *P. longum*; CPL: Chloroform *P. longum*. Values with different superscripts vary significantly within the column (P<0.05)

Table 3: Effect of methanolic extract and fractions of Piper longum on larvae by 6th and 12th h post exposure, (% mortality)

Concentration (mg/mL)	Treatments					
	MPL		n-HPL		CPL	
	6th hour	12th hour	6th hour	12th hour	6th hour	12th hour
1.95	54.55[a]	56.36[a]	45[a]	52.5[a]	52.31[a]	53.85[a]
3.91	78.57[b]	83.93[b]	63[b]	69[b]	50[b]	55[a]
7.81	86.05[c]	86.05[c]	66.94[c]	75.81[c]	63.33[c]	70[b]
15.63	95.12[d]	95.12[d]	65[d]	69.17[b]	58.33[d]	63.33[c]
31.25	95.83[d]	97.92[e]	76.25[e]	81.25[d]	76.92[e]	83.08[d]
62.5	94.12[d]	100[f]	86[f]	89[e]	93.75[f]	95[e]
125	100[e]	100[f]	90[g]	92.5[f]	97.33[g]	100[f]
250	100[e]	100[f]	98.75[h]	100[g]	100[h]	100[f]
500	100[e]	100[f]	100[h]	100[g]	100[h]	100[f]
Pooled SE	0.989	0.807	0.9	0.790	1.008	0.949

Control	
6.25 % Tween 80 in water	2.9
Ivermectin (10 µg/mL)	100
Thiabendazole (10 µg/mL)	100

Note: MPL: Methanolic *P. longum*; n-HPL: n-Hexane *P. longum*; CPL: Chloroform *P. longum*.

Values with different superscripts vary significantly within the column (P<0.05)

Table 4: Effect of methanolic extract and fractions of *Piper longum* on larval migration, (% inhibition).

Concentration (mg/mL)	Treatments		
	MPL	n-HPL	CPL
1.95	44.44[a]	44.23[a]	48.98[a]
3.91	46.27[a]	57.14[b]	67.74[b]
7.81	60.98[b]	69.12[c]	78.95[c]
15.63	57.69[c]	72.92[d]	82.61[d]
31.25	64.71[d]	83.33[e]	88.24[e]
62.5	72.09[e]	82.43[e]	97.83[f]
125	72.5[e]	89.58[f]	95.24[f]
250	83.78[f]	87.04[f]	97.22[f]
500	89.66[g]	92.73[g]	95.24[f]

Control	
6.25 % Tween 80 in water	0
Ivermectin (10 µg/mL)	77.53
Thiabendazole (10 µg/mL)	93.65

Pooled SE: 1.370

Note: MPL: Methanolic *P. longum*; n-HPL: n -Hexane *P. longum*; CPL: Chloroform *P. longum*

Values with different superscripts vary significantly within the column (P<0.05)

study. The IC_{50} for chloroform fraction of *P. longum* was consistently low in EHA, LMA and LMIA with IC_{50} of 1.554, 2.324 and 1.796 respectively indicating its broad spectrum of activity. Hence the methanolic extract and its chloroform fraction was subjected for GCMS and FTIR analysis.

FTIR analysis

The results obtained from FTIR analysis is given in Figure 2 and Table 7.

GCMS analysis

Phytochemicals identified by GCMS and the spectrum obtained Figure 3 for methanolic *P. longum* is given below with their probability and respective retention time Table 8.

Acute Oral Toxicity Study

No mortality was observed among rats administered with, methanolic extract of *P. longum* throughout the study period of 14 days. The rats in the study did not reveal any major chemical adverse effects post dose administration and in subsequent 14 days of the study period except for clinical signs of hypoactivity and mild discomfort for short duration. Rats sacrificed at the end of study period showed mild gross changes in liver at necropsy. The histopathology of the hepatic tissue revealed mild vascular changes.

DISCUSSION

The methanolic extract and its n-hexane and chloroform fractions of *P. longum* possessed promising anthelmintic activity up to the lowest concentration (1.95 mg/mL) employed causing more than 50 per cent inhibition of hatching. The methanolic extract of *P. longum* was highly

potent followed by its hexane and chloroform fraction. The results obtained in larval motility assay indicated that the per cent of larval mortality increased with time and dose. The methanolic extract and n-hexane and chloroform fractions of *P. longum* was efficient causing complete larval mortality at highest concentration of 500 mg/mL within six hours and more than 50 per cent mortality of larvae even at lowest concentration (1.95 mg/mL) by 12th h. From the IC_{50} values, The IC_{50} for methanolic extract and n-hexane fraction of *P. longum* were in the range of 1.29-1.53 mg/mL. The chloroform fraction of *P. longum* possessed lowest activity against L_3 larvae with IC_{50} of 12.47 mg/mL.

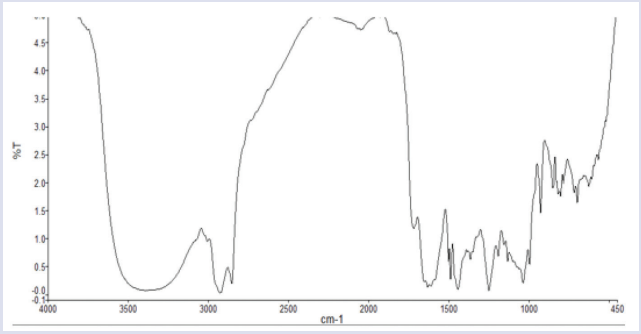

Figure 2: FTIR spectrum of methanolic extract of Piper longum.

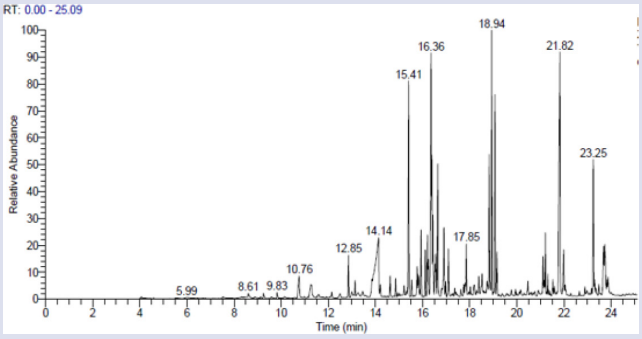

Figure 3: GCMS spectrum for methanolic extract of Piper longum.

Table 5: Minimum concentration and time producing 50 per cent and 100 per cent mortality of adult amphistomes, (mg/mL and minutes)

Extract/ Fraction	50 % Mortality		100 % Mortality	
	Concentration (mg/mL)	Time (minutes)	Concentration (mg/mL)	Time (minutes)
Methanolic	1.95	105	7.81	85
n-hexane	15.63	115	31.25	120
Chloroform	7.81	105	15.63	110
Thiabendazole Control	10 µg/mL	30	10 µg/mL	45
Tyrodes Control	-	120	-	150
6.25 % Tween 80	-	120	-	150

Table 6: Inhibitory concentration-50 (IC50) based on *in-vitro* studies, (mg/mL).

Extract	EHA	LMA	LMIA	AMA
MPL	0.026	1.533	4.412	5.493
n-HPL	0.521	1.383	2.301	105.1
CPL	1.554	2.324	1.796	21.91

Note: MPL: Methanolic *P. longum*; n-HPL: n -Hexane *P. longum*; CPL: Chloroform *P. longum*

Table 7: List of structurally similar compounds for methanolic extract of *Piper longum* as obtained by FTIR analysis.

Potent extract / fraction	Structurally similar compounds
Methanolic extract	Piperine, Beta-estradiol, Farnesyl Acetate, (-)-(1r)-menthyl Acetate, D(+)-galacturonic Acid, Dimethyl Azelate, Piperonylamine, C16-juvenile Hormone, Barbituric Acid, Bis(2-ethylhexyl) Sebacate.

Table 8: List of compounds from methanolic extract of *Piper longum* on GCMS analysis.

Compounds	Retention time (minutes)	Probability	Compounds	Retention time (minutes)	Probability
1-Butanol	4.25	62.86	Benzenepropanoic acid	13.91	42.62
1-Butanol	4.37	51.15	Hydrocinnamic acid	14.13	47.27
2-Thiazolidinecarboxamide	5.99	49.10	Methyleugenol	14.94	73.09
2,4-Dihydroxy-2,5-dimethyl-3(2H)-furan-3-one	7.52	89.09	Spathulenol	17.75	45.42
Benzyl alcohol	8.61	47.47	Caryophyllene oxide	17.85	45.42
Benzenemethanol	9.13	44.17	Spathulenol	18.39	70.08
1,6-Octadien-3-ol	9.83	71.74	Hexadecanoic acid	21.59	73
4H-Pyran-4-one	10.76	93.74	n-Hexadecanoic acid	21.98	46.35
2H-Pyrrol-2-one	11.26	60.94	2,4-Pentadienoic acid	22.65	62.22
2-Piperidinone	11.58	84.62	2,4-Pentadienoic acid	23.25	70.32
N-Ethylhexahydro-1-Hazepine	11.80	43.92	9,12,15-Octadecatrienoic acid	24.43	45.17
Benzenepropanoic acid	12.85	62.74	Benzenepropanoic acid		

The methanolic extract, n-hexane and chloroform fractions of *P. longum* were effective in inhibiting larval migration even at lowest concentration (1.95 mg/mL) with inhibition of 89.66, 92.73 and 95.24 per cent respectively. From the IC_{50} values, chloroform fraction of *P. longum* was most potent in inhibiting larval migration (IC_{50}= 1.796 mg/mL) followed by n-hexane fraction and methanolic extract of *P. longum* with IC_{50} of 2.301 and 4.412 mg/mL respectively. The results of adult mortality assay indicated that all the extract/fractions possessed activity against adult amphistomes. The methanolic extract of *P. longum* caused 50 per cent mortality of amphistomes at 1.95 mg/mL and complete mortality at 7.81 mg/mL. The chloroform fraction of *P. longum* was equally effective causing 50 per cent mortality at 7.81 mg/mL and 100 per cent mortality of adults at 15.63 mg/mL respectively. The n-hexane fraction of *P. longum* caused 50 per cent mortality at 15.63 mg/mL while complete mortality was observed at concentration of 31.25 mg/mL. The IC_{50} data obtained in the study suggested that the methanolic extract of *P. longum* fruit was highly potent (IC_{50} = 5.493 mg/mL) followed by chloroform and hexane fractions with IC_{50} of 21.91 and 105 mg/mL respectively.

The extract/fraction can be effective as anthelmintic if it produces more than 90 per cent inhibition of egg hatching.[26] The phytochemicals like tannins, phenols, flavonoids, sterols etc. present in the extracts and fractions of *P. longum* contributed to the anthelmintic potency of the fruits.[27] Though the exact mechanism of how these plant chemicals because anthelmintic activity has not been established, certain hypothesis had been predicted. Destabilization of egg membranes led to penetration of plant extract into the egg and caused either embryonic mortality or prevented development of embryo within the egg.[28]

The polyphenolic compounds tannins were present in the methanolic extract of n-hexane and chloroform fractions of *P. longum,* interfered with energy generation in the nematode. Tannins cause mortality of the parasite by binding to the glycoprotein on the tegument of the parasite.[29]

The presence of terpenoids in methanolic extract of n-hexane and chloroform fractions of *P. longum* were considered to cause inhibition of egg hatching synergistically with other phytochemicals including alkaloids, tannins, flavonoids etc. Phenols and flavonoids were found in the methanolic extract of n-hexane and chloroform fractions of *P. longum* which also contributed to anthelmintic activity. The results are in accordance with the reports of Saddiqe *et al.*[25] who stated that phenols and flavonoids were the major components in *S. nigrum* contributing to anthelmintic potency of the plant. The flavonoid, terpenoid, and steroid rich ethanolic and dichloromethane extract of *Phytolacca icosandra* caused inhibition of larval motility and caused death of larvae.[31] as reported in the present study.

The presence of terpenoids in methanolic extract, n-hexane and chloroform fractions of *P. longum* can be considered to cause death of amphistomes synergistically with other phytochemicals. Enwerem *et al.*[32] reported that adulticidal activity of terpenoids in *Berlina grandiflora* against *Caenorhabditis elegans*

Iqbal *et al*.[33] reported that the adulticidal activity of *Zingiber officinale, Allium sativum and Ficus religiosa* against *H.* contortus which was attributed to the presence of potent phytochemicals in the plants. Tannins are polyphenolic compounds which caused mortality of adult amphistomes, probably by its interaction with glycoprotein and cause functional disintegration of this layer.[30]

The GCMS analysis revealed the presence of piperidinone, hydrocinnamic acid, ethylhexahydro azepine, methyleugenol, hexadecanoic acid and caryophyllene oxide were identified in the methanolic extract of *P. longum* while octadecatrienoic acid, caryophyllene, caryophyllene oxide and hydrocinnamic acid were present in the chloroform fraction of *P. longum*. Similar compounds were isolated by Liu *et al.*[23] from plants belonging to piperaceae family including spathulenol, piperitone, eugenol and caryophyllene. Mohammed *et al.*[34] isolated piperidine, benzofuran, spathulenol, pyrrolizin from *P. nigrum* which are in support to the present finding.

Anthelmintic activity of caryophyllene oxide in essential oils of aerial parts of *Artemisia campestris* spp, carboxylic acids (acetic acid, butanoic acid, hexadecanoic acid and propanoic acid), 1-monolinoleoylglycerol trimethylsilyl ether, caryophyllene, tetradecanoic acid, 9,12,15-octadecatrienoic acid and 2- pentanone from leaf of *Melia composite* and octadecanoic acid, hexadecanoic acid and sesquiterpenes from *Cleome chelidonii* has been reported previously.[35,36,37,38]

CONCLUSION

From the results of the study, it could be concluded that the methanolic extract and fractions of *P. longum* possessed potent anthelmintic activity. The chloroform fraction of *P. longum* possessed potent broad spectrum anthelmintic activity as it was effective against strongyle ova, larva and adult amphistomes at low IC_{50} values. Though the extracts possessed mild toxicity, the dose required for anthelmintic activity was very less and isolation of the active compounds can provide a lead for the development of a novel and safe anthelmintic.

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CONFLICT OF INTEREST

We declare that we have no conflict of interest.

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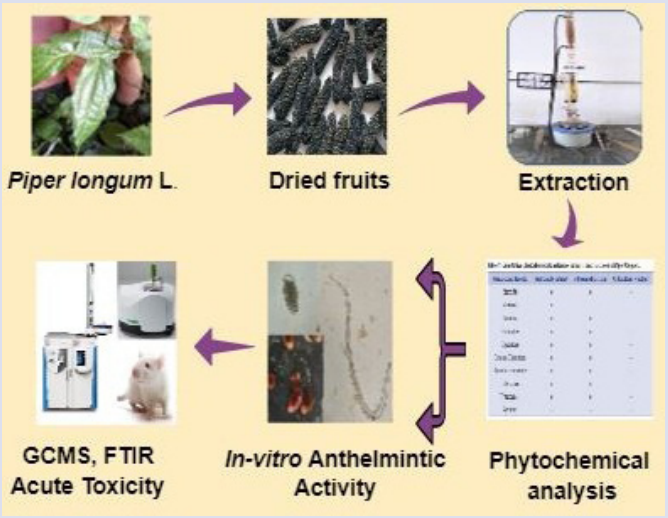
SUMMARY

- Gastrointestinal infections among livestock are gaining much importance not merely due to its occurrence, but also due to wide spread anthelmintic resistance. Phytochemicals are ideal alternatives to combat the infection and resistance. The present study was conducted with the view to evaluate the *in vitro* ovicidal (EHA), larvicidal (LMA and LMIA) and adulticidal activity of methanolic extract and various fractions from fruits of *Piper longum* against strongyle ova, larvae and adult amphistomes. Fruits were collected, identified, shade dried and extracted with methanol and the extract was successively fractionated using different solvents. The extracts and fractions were diluted serially in 6.25 per cent tween 80 to obtain concentrations of 500, 250, 125, 62.5, 31.25, 15.63, 7.81, 3.91 and 1.95 mg/mL. Ivermectin and thiabendazole at 10 µg/mL acted as positive control and the solvents as negative control. The *in vitro* anthelmintic activity was performed. IC_{50} was calculated and GCMS, FTIR analysis and acute oral toxicity study (OECD No. 420) of potent extract/fraction was performed to know the structure of active ingredient.
- The fruits were identified and accession number 006 was obtained. The phytochemical analysis revealed the presence of flavonoids in all the extract and fractions while saponins were absent. All extract and fractions possessed *in vitro* anthelmintic activity. The methanolic extract was potent (IC_{50} of 0.026 mg/mL) against strongyle ova while the chloroform fraction possessed potent activity on larval migration (IC_{50}= 1.796 mg/mL). The methanolic extract was potent against adult amphistomes (IC_{50} of 5.493 mg/mL) causing morphological changes in tegument, syncytium and parenchyma on histopathology. GCMS analysis of potent methanolic extract revealed the presence of piperidinone, hydrocinnamic acid, caryophyllene oxide etc. No observable clinical signs were detected during the acute oral toxicity study except initial hypo activity, pale discoloration of liver on post mortem and on histopathology mild vascular changes in liver was noticed.
- From the results of the study it could be concluded that the chloroform fraction of *P. longum* possessed broad spectrum anthelmintic activity as it was effective against strongyle ova, larva and adult amphistoes. Isolation of the active compounds can provide a lead for the development of a novel, safe anthelmintic which may have a novel mechanism of action.

GRAPHICAL ABSTRACT



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