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Studies on the nematicidal potentials of *Pleurolobus gangeticus* and *Tragia involucrata*

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The study aimed at exploring and validating the nematicidal potentials of *Pleurolobus gangeticus* and *Tragia involucrata* against the gastrointestinal parasitic nematode of cattle called *Haemonchus contortus*. Both plants are well known for their ethno-pharmacological properties and have been used by various traditional healers. Plant parts were extracted using ethyl acetate, ethanol and water in an accelerated solvent extractor and the crude extracts were evaluated for its nematicidal potentials against eggs and L3 larvae of *H. contortus* using egg hatch inhibition assay and larval paralysis assays respectively. The effects of the potent extracts on mitotic machinery was evaluated *in vitro* by performing an *Allium cepa* assay. Molecular docking analysis was also performed to evaluate the binding affinity of phytoconstituents to tubulin protein. The ethanol extracts of both *P. gangeticus* (DME) and *T. involucrata* (TME) showed the strongest inhibition of 90.83±0.98% and 90.5±1.64% respectively on hatching of eggs at 10 mg/mL concentration. At this concentration, larval paralysis assay also showed 65.5±2.25% mortality for DME and 64.5±0.54% mortality for TME treated nematodes. Ethyl acetate extracts of both plants showed relatively less inhibition on egg hatch and larval paralysis and the aqueous extracts were the least potent among the three. The *Allium cepa* assay revealed an accumulation of cells in the metaphase when treated with colchicine (32.3%) or extracts (DME-36.1%; TME-35.5%) as compared to the vehicle controls (22.9%). *In silico* analysis revealed that several components in DME and TME has strong binding affinity with the colchicine binding site (CBS) of tubulin protein complementing its observed anthelmintic potential.

Keywords: Anthelmintics, Ethnopharmacology, *Haemonchus contortus*, Molecular docking, Tubulin

The gastrointestinal parasites of ruminants clinically take a toll on their hosts and lead to severe economic loss in the livestock industry. Each portion of the digestive system of the hosts is occupied by specific genera of nematodes. The abomasum is usually attacked by Haemonchus, Ostertagia, Teladorsagia and Trichostrongylus. Cooperia, Nematodirus and Trichostrongylus are seen in the small intestine and Oesophagostomum in the large intestine[1,2]. Haemonchosis caused by *Haemonchus contortus* (Family, Trichostrongylidae ; Order, Rhabditida), results severe anemia in cattle due to its blood sucking activity and ultimately reduces the growth of animals, affects milk production and may also lead to death in severe untreated cases[3]. Resistance towards anthelmintic drugs have become a major cause of concern as it is both heritable and rarely reversible. Several genetic and biological factors like short life cycle, increased reproductive rates, large population and quick evolution in nematodes have made them less vulnerable to the synthetic anthelmintic drugs; *H. contortus* being the most resistant among them[4]. The use of secondary metabolites derived from plants as a source of anthelmintic drug is one of the best and most effective alternative methods to fight anthelmintic resistance in nematodes[5].

Pleurolobus gangeticus (L.) J.St.-Hil. Ex H. Ohashi & K. Ohashi (synonym *Desmodium gangeticum*) of Fabaceae Family, is of great medicinal importance and is used as a febrifuge, anticatarrhal, antiemetic, anti-inflammatory agent and also for digestive problems. It is also an important ingredient of an ayurvedic preparation called ‘dashamoolakwaath’ and ‘dashamoolarishta’ used during postnatal care to avoid secondary complications[6,7]. *Tragia involucrata* Linn of Euphorbiaceae Family has been used against inflammation, allergy, epilepsy, renal stones, asthma, bronchitis, vomiting, diarrhea and numerous other ailments in ethnomedicine practices. The plant has mention in the scriptures of Ayurveda like *Charaka samhita*, *Sushruta samhita* and *Vagbhata samhita* by the name ‘Vrishchakali’, for the treatment of epilepsy, fever and respiratory tract disorders[8]. In this study we evaluated the anthelmintic potential of ethyl acetate, ethanol and aqueous extracts of *P. gangeticus* and *T. involucrata* on eggs and L3 larvae of *H. contortus* isolated from goat fecal samples. *In silico* analysis on the interactions of bioactive molecules identified from the extract to that of the colchicine binding sites (CBS) of nematode tubulin was also part of this study.

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$$x = \frac{-b \pm \sqrt{b^2 - 4ac}}{2a}$$

Materials and Methods

Plant collection, authentication and extraction

P. gangeticus and *T. involucrata* were collected from different parts of Kerala, India and identified at Kerala Forest Research (KFRI), Kerala, India. Voucher specimens with accession numbers 17683 and 17682 respectively are kept in their herbarium. Plants (stem and roots for *P. gangeticus* and whole plant for *T. involucrata*) were shade dried, powdered and extracted using ethyl acetate, ethanol or water in an accelerated solvent extractor, dried using a vacuum evaporator and stored under refrigeration until further use.

Egg hatch assay (EHA)

The egg hatch assay was conducted according to the World Association for Advancement of Veterinary Parasitology (WAAVP) guidelines[9]. Serially diluted (0.625, 1.25, 2.5, 5 and 10 mg/mL) extracts were added to each of the 6 well plates in triplicates. Albendazole (6.25, 12.5, 25, 50 µg/mL) was maintained as the standard reference, while 1% DMSO served as the vehicle control. Approximately 100 eggs in 0.5 mL distilled water was added to each of this well. All the multi well plates were incubated at 27°C for 48 h. After 48 h of treatment, hatching was inhibited by adding Lugol's iodine solution. The unhatched eggs and hatched larvae (dead or alive) were counted using a stereozoom microscope (40× magnification).

Larval paralysis assay (LPA)

For LPA, the protocol previously described by Varady & Corba (1999) was used with slight modification[10]. Fecal sampling of naturally infected goats was done and it was cultured at 27°C for a week to obtain third instar *H. contortus* larvae. Approximately 100 motile larvae in 500 µL water were prepared, and to this equal volumes of plant extracts in concentrations 0.625, 1.25, 2.5, 5 and 10 mg/mL were added in triplicates. Albendazole (6.25, 12.5, 25, 50 µg/mL) was kept as the reference drug and 1% DMSO served as vehicle control. All samples were then incubated at room temperature for 24 h. After 24 h of treatment, the loss of motility of the larvae were recorded. A stereozoom microscope at 40× magnification was used to count non-motile larvae and the results are expressed as Mean ±SD.

Metaphase indexing in *Allium cepa*

Allium cepa root tip meristematic cells are used extensively for screening the effect of antimitotic drugs. This assay was used to analyse the effect of DME and TME on mitosis. The experiment was conducted according to Chakraborty *et al*. (2021) with minor modifications[11]. Onion bulbs were collected locally and post removal of the outer scales, the onion bulbs were placed in distilled water overnight at room temperature (27-28°C) for it to germinate. The germinated bulbs were then treated with either colchicine 0.4 mg/mL (reference), 1% DMSO (vehicle control), DME (4 mg/mL) or TME (4 mg/mL) for a period of 4 h. After 4 h, 1N HCl was used for hydrolysing the roots. Staining was done with 1% toluidine blue following the standard protocol and the meristematic region was visualised under a microscope (40× magnification) for different stages of mitotic phase[12,13]. The metaphase index was calculated using the formula:

$$\text{Metaphase index} = \frac{\text{No of cells in meta}}{\text{Total No.of cells in division}} \times 100$$

In silico studies

Molecular docking studies were carried out using the AutodockVina 1.5.7. AutoDock Vina, an open source program designed and developed by Oleg Trott, was used for conducting molecular docking studies[14]. The 3D structure of all the ligands was retrieved from PubChem and that of the proteins were retrieved from RCSB-PDB (PDB ID: 6E88). A modelled α-β tubulin of *H. contortus* (HcF) was also used for comparison. The CBS of alpha-beta tubulin was selected as the target site and the reference drugs, albendazole mebendazole and colchicine were initially used for docking studies. The studies were then repeated using the phytoconstituents identified in DME and TME.

Statistical analysis

SPSS software version 24 was used for probit analysis to calculate the effective concentration required to induce 50% (EC_{50}) and 90% (EC_{90}) inhibition to nematode egg hatch and L3 larval paralysis. Student's *t*-test was used comparison of extracts treated animals with vehicle controls. The P value ≤ 0.05 was considered significant.

Results and Discussion

Egg hatch assay (EHA)

A dose dependent inhibition on egg hatch was observed as shown in Fig. 1 with the aqueous extracts (DWE and TWE) being the least active. At the

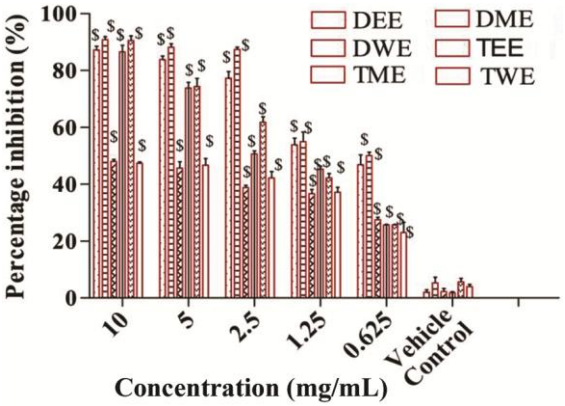

Fig. 1 — Inhibition of *H. contortus* eggs hatch with treatment of *P. gangeticus* and *T. involucrata* extracts; All the extract treated groups were individually compared with vehicle controls $P \leq 0.001$ ($).

Table 1 — EC_{50} and EC_{90} values (mg/mL) on the egg hatch inhibition by different extracts

Extracts Used	EC_{50}	EC_{90}
DEE	0.771	>10
DME	0.661	6.407
DWE	>10	>10
TEE	1.827	>10
TME	1.664	>10
TWE	8.760	>10
Albendazole	0.727	>10

highest tested concentration (10 mg/mL) DEE and DME showed strong egg hatch inhibition of 87.16±1.32% and 90.83±0.98% respectively. Similarly, TEE and TME also showed strong egg hatch inhibition of 86.5±2.34% and 90.5±1.64% respectively. The EC_{50} value of egg hatch inhibition was calculated (Table 1) and DME showed an EC_{50} of 0.661 mg/mL which was the lowest when compared to DEE (0.771 mg/mL) and DWE (>10 mg/mL). Similarly, with the lowest EC_{50} value of 1.664 mg/mL for TME seemed to be the most potent when compared to TEE (1.827 mg/mL) and TWE (8.766 mg/mL).

Larval paralysis assay (LPA)

The paralysis of L3 larvae after treatment with extracts of *P. gangeticus* and *T. involucrata* are shown in Fig. 2. At the highest tested concentration (10 mg/mL) DME and TME induced 65.5±2.25% and 64.5±0.54% of larval paralysis. Further, at the same concentration, DEE and TEE showed 57.5±2.5% and 60±4.42% larval paralysis respectively. The least effect on paralysis of L3 larva at 10 mg/mL was shown by the aqueous extracts DWE (39.33±0.81%) and TWE (48.83±0.4%). The lowest EC_{50} values were observed

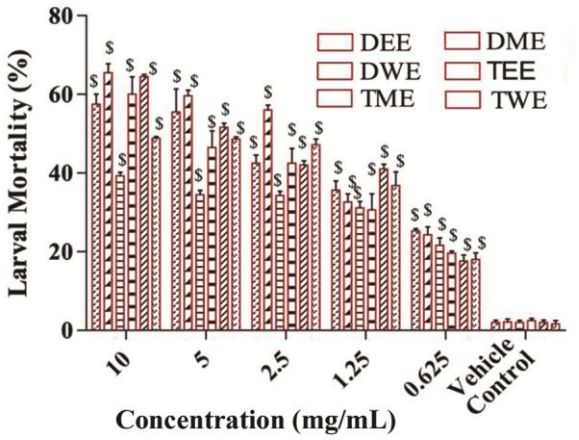

Fig. 2 — Larval paralysis of *H. contortus* L3 larvae by the treatment of *P. gangeticus* and *T. inolucrata* extracts. All the extract treated groups were individually compared with vehicle controls $P \leq 0.001$ ($).

Table 2 — EC_{50} and EC_{90} values in mg/mL on the larval paralysis by different extracts

Extracts Used	EC_{50}	EC_{90}
DEE	4.370	>10
DME	2.931	>10
DWE	>10	>10
TEE	5.166	>10
TME	3.957	>10
TWE	6.412	>10
Albendazole	0.306	8.639

for DME (2.931 mg/mL) and TME (3.957 mg/mL) and thus were considered the most potent extracts in causing paralysis of L3 larvae. DEE and TEE had EC_{50} values of 4.370 and 5.166 mg/mL respectively and the EC_{50} values of DWE (>10 mg/mL) and TWE (6.412 mg/mL) were the highest among others and thus were considered as the least potent in causing larval paralysis (Table 2).

Metaphase indexing in *Allium cepa*

In the *Allium cepa* assay, as expected, the colchicine treated root tips showed a higher metaphase index of 32.3 when compared to vehicle treated samples (22.9). However, when compared to colchicine treated root tips, DME (36.1) and TME (35.5) treated samples showed higher metaphase indices (Table 3). An increase in the percentage of cells in prophase and metaphase stages and a decrease in the anaphase and telophase stages in the treated groups were observed as shown in Fig. 3.

***In silico* studies**

Several compounds showed higher binding affinity towards CBS of *C. elegans* and *H. contortus* as compared to the reference drugs albendazole and

Table 3 — Effect of DME and TME on dividing cells

Treatment	Total number of cells	Total number of cells in division				Metaphase index
		Prophase	Metaphase	Anaphase	Telophase	
Control (1% DMSO)	200	5.33±0.57	6.66±1.15	7.33±1.52	9.66±0.57	22.9
Colchicine (0.4 mg/mL)	200	8.66±1.15	10.66±1.15	7.33±0.57	6.33±0.57	32.3
DME (4 mg/mL)	200	9.33±0.57	11.33±0.57	6.33±0.57	4.33±0.57	36.1
TME (4mg/mL)	200	10.33±0.57	12.33±0.57	6.33±0.57	5.66±1.15	35.5

[Data expressed as Mean±SD]

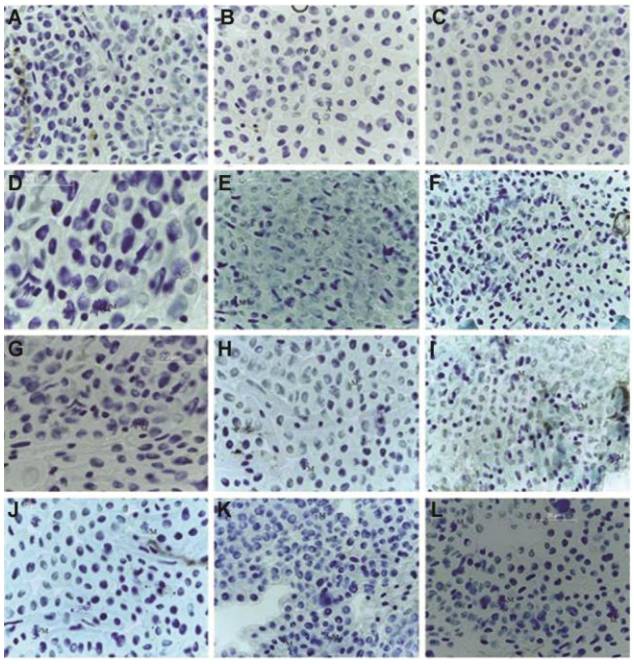

Fig. 3 — Photomicrographs of representative meristematic cells of *Allium cepa* root tips after exposure to vehicle, colchicine, DME and TME for 4 h showing various stages of mitosis namely; prophase (P), metaphase (M), anaphase (A) and telophase (T). A, B and C; shows cells of root tips treated with vehicle control. D, E and F; shows cells of root tips treated in colchicine. G, H and I; shows cells of root tips treated in DME. J, K and L; shows cells of root tips treated in TME.

mebendazole (Table 4). The binding affinity of colchicine against the CBS of *C. elegans* (-8.6 Kcal/mol) and *H. contortus* (-8.2 Kcal/mol) was even lower than mebendazole. Lupeol, schaftoside, quercetin and thymol are also known to bind with CBS with high affinities. All these molecules except thymol showed strong binding affinity towards the CBS of *C. elegans* and *H. contortus* that ranged between -8.7/-8.5 – - 9.8/-10.9 Kcal/mol. However, thymol had a low binding affinity of -6.0 Kcal/mol and -6.1 Kcal/mol towards the CBS of *C. elegans* and *H. contortus* tubulin respectively. Apart from the aforementioned molecules several others detected via LC-MS in DME and TME had strong binding affinity towards CBS of *C. elegans* and *H. contortus* tubulin when compared to colchicine.

Table 4 — Binding affinity (Kcal/mol) of selected ligands on colchicine binding site of tubulin

Ligands	Binding affinity (Kcal/mol)	
	6e88	HcF
Reference Molecules		
Colchicine	-8.6	-8.2
Albendazole	-7.3	-7.1
Mebendazole	-9.1	-8.8
Previously reported molecules		
Thymol	-6.0	-6.1
Lupeol	-8.9	-8.5
Quercetin	-8.7	-8.8
Schaftoside	-9.8	-10.9
Molecules detected in DME and TME		
Isoquercetin	-9.9	-9.6
Corilagin	-9.5	-9.8
Echinacin	-10.9	-10.0
Agathisflavone	-9.7	-10.2
Apigetrin	-10.7	-10.6
Cynaroside	-10.8	-10.7
Leufolin A	-10.0	-11.2

C*aenorhabditis elegans* is a closely related nematode to *H. contortus* and the third larval stage (L_3) is also known to be similar in both[15]. The pairwise sequence alignment also showed that the alpha and beta tubulin of *C. elegans* and *H. contortus* has a similarity of 87.11% and 93.6% respectively. Considering the similarity, we used *C. elegans* tubulin structure (PDB ID: 6E88) as template for predicting the 3D structure of *H. contortus* tubulins. The binding affinity of the reference molecules, previously reported tubulin binding phytoconstituents and the secondary metabolites identified in the *P. gangeticus* and *T. involucrata* are detailed in Table 4[16,17]. As enlisted in our previous studies DME has several pharmacologically important molecules including lupeol, thymol and schaftoside[16]. Similarly, in TME also several biologically active secondary metabolites were detected *viz*; corilagin, loquatoside, cynaroside, isoquercetin, apigetrin, leufolin A, agathisflavone, quercetin and echinacin in our previous study[17].

Lupeol detected in DME have been previously reported to have interactions with mebendazole binding site of β tubulin and had been proposed to be the probable mechanism behind its anthelmintic potentials[18]. Similarly, in another *in silico* study thymol showed strong inhibitory effects on β tubulin isotype-1 of *H. contortus* compared to albendazole[19]. It was also observed in this study that, both thymol and albendazole shared the same 'ligand binding site' though the orientation of interacting amino acid residues were different. Schaftoside, a flavonoid molecule identified in DME have also shown strong binding affinity towards β tubulin (5IJ0 based model) of pine wood nematode *Bursaphelenchus xylophilus*[20]. In all the three studies mentioned above, molecular docking studies of the ligands were done against β tubulin protein of various organisms. In the case of lupeol, molecular docking was carried out with *Bos taurus* β-tubulin (PDB ID: 7ODN) which has only 85.6% sequence similarity with *H. contortus* β tubulin. In the case of thymol and albendazole, they used a modelled 3D structure of *H. contortus* β tubulin (PDB ID: 1OJ0) but the binding affinity was much less compared to other active molecules and reference compound albendazole. This could also be attributed to the 'ligand binding site' selected in this study rather than using CBS. In the *in silico* analysis involving schaftoside, the target β tubulin was modelled using 5IJ0 (a microtubule assemble from human tubulin TUBB 3) as template owing to its sequence similarity of 86.38%.

CBS stands out among other binding sites in tubulin owing to its unique ability to bind to small molecules and overcome drug resistance[21]. Colchicine is known to block mitotic cells in metaphase by forming a tubulin-colchicine complex, thereby inhibiting the elongation of microtubule polymers[22]. Consistent with these, the *Allium cepa* assay we conducted showed an accumulation of dividing cells in the metaphase when treated with colchicine or extracts as compared to the vehicle controls (Table 4). Initially we repeated the interaction studies with colchicine and CBS of *C. elegans* tubulin (PDB ID: 6E88) and a modelled *H. contortus* α-β tubulin *viz.* HcF that gave consistent results. Additionally, lupeol detected in DME had a strong binding affinity with both *C. elegans* (-8.9 Kcal/mol) and *H. contortus* (-8.5 Kcal/mol) CBS. Similarly, Schaftoside also showed strong binding affinity towards *C. elegans* tubulin (-9.8 Kcal/mol) and HcF (-10.9 Kcal/mol). Quercetin detected in TME also had high binding affinity with the tubulin CBS of *C. elegans* (-8.7 Kcal/mol) and *H. contortus* (-8.8 Kcal/mol). This was also consistent with an earlier study that reports the tubulin binding efficacy of this compound identified from ethanolic extract of *Kaempferia rotunda*[23]. On the contrary, thymol showed a low binding affinity towards the tubulin protein of *C. elegans* (-6.0 Kcal/mol) as well as HcF (-6.1 Kcal/mol).

Apart from these compounds with previously known tubulin interactions, several other molecules present in TME *viz*; Isoquercetin, Cynaroside, Apigetrin, Leufolin A, Loquatoside, Agathisflavone and Echinacin also showed very high binding affinity towards the CBS of *C. elegans* as well as HcF tubulin. It was further interesting to note that several of these compounds had a higher binding affinity when compared to reference drugs used in the *in silico* study (Table 4).

Conclusion

Since a diverse range of phytochemicals in DME and TME were found to have a great binding affinity towards the tubulin protein of nematodes, it may be noted that the synergestic effect of these phytocomponents may be the reason for tubulin depolymerisation followed by mitotic arrest, and nematicidal activity. Fractionation and isolation of these specific components may be required to make more conclusive findings.

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Author contribution

All authors have read and approved the manuscript. MM conduct of experiments, drafting manuscript, DM conduct of experiment, LV design of the study, critical reading and finalising the manuscript.

Conflict of interest

The authors have no conflicts of interest to declare.

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