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ORIGINAL ARTICLE

In vitro and in vivo anthelmintic study of *Sesbania sesban* var. *bicolor*, a traditionally used medicinal plant of Santhal tribe in Assam, India

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Abstract The leaf decoction of *Sesbania sesban* var. *bicolor* is used traditionally by Santhal tribe of Assam, India, for the treatment of intestinal helminthic infections. This study was conducted to evaluate the in vitro and in vivo anthelmintic efficacy of methanolic extract of *S. sesban* var. *bicolor* leaves using *Hymenolepis diminuta*-rat (cestode) and *Syphacia obvelata*-mice (nematode) as test parasites and models. Praziquantel (PZQ) and albendazole (ABZ) were used as reference drugs. At the highest concentration of 30 mg/ml of the plant extract, *H. diminuta* and *S. obvelata* showed mortality at 0.81 ± 0.01 h and 15.17 ± 0.05 h, respectively. The in vivo results substantiated the in vitro findings, and the extract showed a better cestocidal efficacy in a dose-dependent manner, whereby treatment of rats with 400 mg/kg of the plant extract caused 65.10% reduction in eggs per gram (EPG) of faeces and 56% reduction in worm counts. *S. obvelata*-infected mice treated at the same dose showed 34.32% and 47.08% reduction in EPG and worm counts at necropsy, respectively. The methanolic extract was subjected to bioassay-guided fractionation using different solvents and the ethyl acetate fraction proved to be the most active. This active fraction was subjected to column chromatography using varying concentrations of hexane:ethyl acetate. Maximum efficacy was observed in 7:3 hexane:ethyl acetate, where *H. diminuta* and *S. obvelata* showed mortality at 3.56 ± 0.12 h and 9.21 ± 0.02 h, respectively. This indicates that the isolated fraction contained the active component responsible for its anthelmintic activity, which substantiates the medicinal usage in traditional practice.

Keywords Active fraction · Anthelmintic · Helminthiasis · *Hymenolepis diminuta* · *Sesbania sesban* var. *bicolor* · *Syphacia obvelata*

Introduction

Intestinal helminthiasis, is an important public health problem in tropical and sub-tropical countries. It affects around 1.5 billion people globally (WHO 2015). At present the control of intestinal worms is based on mass drug treatment by two common drugs, albendazole and mebendazole. However, in some areas of the world synthetic medicines are still out of reach and therefore, alternative strategies developed from traditional knowledge to combat such diseases have emerged since times immemorial (Deori and Yadav 2016).

Sesbania sesban (L.) Merr. var. *bicolor* (Wight & Arn.) F. W. Andrews (Fabaceae) (Fig. 1), commonly called as "Mondormoli" in the Santhali language, is widely distributed and cultivated throughout semi-arid and sub-humid tropical regions (Göhl 1982). It is a small perennial tree and is very common throughout Africa and in Asian countries like India, Malaysia, Indonesia and the Philippines (Nigussie and Alemayehu 2013; Kumar et al. 2014). Five varieties of *S. sesban* have been recognised botanically (Gutteridge 1993; Mani et al. 2011) viz., *S. sesban* var. *sesban*, *S. sesban* var. *bicolor*, *S. sesban* var. *nubica*, *S. sesban* var. *zambesiaca* and *S. sesban* sub sp. *punctata*.

In traditional medicine, various parts of this plant are used as an anti-inflammatory, anthelmintic, anti-diarrheal,

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Fig. 1 *Sesbania sesban* var. *bicolor* **a** twig **b** flower **c** leaves being made into pellets

and anti-oxidant (Gomase et al. 2012; Goswami et al. 2016; Murugan and Muniyappan 2018). The aqueous extract of the leaves was shown to possess anti-diabetic properties in a study on normal and streptozotocin-induced diabetic rats (Pandhare et al. 2011). Kamel et al. (2011) reported a moderate in vitro effect of the methanolic extract of *S. sesban* against *Schistosoma mansoni*. Likewise, Subramanian and Kalava (2014) reported the anti-inflammatory effects of the aqueous extract of the seed in carrageenan-induced paw edema model in rats. Similarly, Ibrahim (1992) reported the in vitro efficacy of its aqueous extract against *Caenorhabditis elegans.* Also, Tatiya et al. (2013) isolated diosgenin and oleanolic acid which showed anti-inflammatory activity in both in vitro and in vivo studies in animal models like carrageenan- and histamine-induced rat paw edema, cotton pellet granuloma, acetic acid-induced vascular permeability and oxazolone-induced delayed-type hypersensitivity. Another study by El-Emam et al. (2015) revealed that treatment of mice infected with *S. mansoni* using the methanol extract along with mefloquine reduced the worm burden. Condensed tannins from *S. sesban* and *Desmodium intortum* have been shown to reduce *Haemonchus contortus* infection in goats (Debela et al. 2012).

The phytochemical analysis of *S. sesban* has shown the presence of alkaloids, proteins, flavonoids and phytosterols in the chloroform, methanol, ethanol and aqueous extracts. Its methanol and ethanol extracts were found to contain phenolic compounds and the aqueous extract showed the presence of saponins. Methanol, ethanol and aqueous extract showed the presence of carbohydrates (Kumar et al. 2014). Also, Kohli (1988) reported a saponin, stigmasta-5, 24(28)-diene-3β-O-β-D-galactopyranoside from the seeds from *S. sesban*. A saponin isolated from the plant identified as 3-O-[α-L-rhamnopyranosyl-(1 → 3)-β-D-glucuronopyranosyl]-oleanolic acid showed molluscicidal activity (Dorsaz et al. 1988).

In many studies to investigate the bioefficacy of plant extracts, most workers have used in vitro methods. However, in vitro studies alone do not validate the anthelmintic efficacy of the plant (Athanasiadou et al. 2007). For example, in some studies the in vitro and in vivo bioefficacy effects of a plant did not supplement each other (Bøgh

et al. 1996; El-Bahy and Bazh 2015). In vitro activity of a plant does not guarantee comparable in vivo effect. Hence, in vitro studies need to be supplemented or validated with in vivo studies (Deori and Yadav 2016). Several active components have been isolated from *S. sesban* by several workers. Anthocyanins isolated were found to have anti-oxidant and anti-microbial activity (Kathiresh et al. 2012). Kumar et al. (2014) showed that the extract of the whole plant possessed campesterol, β-sitosterol, cyanidine, del-phinidin glycosides, α-keto glutaric acid, oxaloacetic acid, pyruvic acid, oleanolic acid, saponins, palmitic acid, stearic acid, oleic acid, linoleic acid, cyanidin and delphinidin glycosides. Also, Singh et al. (2017) isolated β-sitosterol and kaempferol from roots and stem of *S. sesban*.

To the best of our knowledge, in vitro and in vivo anthelmintic activity of this plant has not been recorded using intestinal helminth parasite models. Based on these facts, this study was undertaken to investigate and validate the anthelmintic efficacy of this plant using in vitro and in vivo assays and also to find its active column fraction.

Materials and methods

Chemicals and drugs

All chemicals and drugs used were of analytical grade. Albendazole (Ambalal Sarabhai Enterprises Ltd., Vado-dara) and praziquantel (Distocide, Chandrabhagat Pharma Pvt. Ltd., Mumbai) were used as reference drugs.

Plant material

A field survey in the Santhal-inhabited districts of Assam, viz, Kokrajhar, Udalguri, Baksa and Chirang, revealed that the local traditional healers prescribe the leaves of *S. ses-ban* var. *bicolor* to treat intestinal helminth infections. The leaves are ground, made into pellets and taken orally by patients having intestinal helminth infections. The plant material was collected from Kokrajhar district of Assam, India, during August and September, 2015. It was identi-fied by a plant taxonomist in the Department of Botany, North-Eastern Hill University (NEHU), Shillong and a voucher specimen (NEHU-12084) was deposited in the herbarium museum of the same department. The leaves were washed with distilled water to remove impurities, dried in shade and then powdered. The powdered material was extracted with methanol in a Soxhlet extractor. The extract (yield 20.21%, w/w) was stored in glass vials at 4 °C.

Phytochemical testing

The extract was subjected to phytochemical testing to confirm the presence of various secondary metabolites. The qualitative analysis was undertaken using the methods of Harborne (1973), Evans (1989) and Sofowora (1993).

Bio-assay guided fractionation

Different fractions of the methanolic crude extract of *S. sesban* var. *bicolor* were separated out using solvents such as hexane, diethyl ether, chloroform, ethyl acetate and n-butanol using a fractionating funnel to obtain different solvent phases. The most active solvent phase was obtained by subjecting each extracted solvent phase to in vitro testings (Gueye et al. 2011). The most active solvent phase was then subjected to silica gel column chromatography to obtain different column fractions using different ratios of hexane:ethyl acetate. The column length was 35 × 3 cm filled with silica gel and mesh size was 60–120. Each column fraction was then subjected to in vitro testings to find the most efficacious fraction (Devi and Muthu 2015).

Experimental animals

Albino rats of both sexes (Wistar strain), infected with *H. diminuta*, weighing about 180–220 g and Swiss albino mice of both sexes, infected with *S. obvelata*, weighing about 25–30 g were used to perform in vivo experiments. The animals were maintained in separate acrylic cages and were given food and water ad libitum. *S. obvelata* infection was identified in mice using the perianal cellophane tape test, as described by Meade and Watson (2014), with slight modifications. *H. diminuta* infection was maintained in rats as described by Tangpu et al. (2006). All experiments on rats and mice were done after due approval from the Institutional Animal Ethics Committee (Animal Models) of North-Eastern Hill University, Shillong. Experimental animals were provided adequate living conditions and procedures were performed with adequate anaesthesia to ensure painless death as per ethical guidelines.

Anthelmintic assay

In vitro tests

Adult live worms of *H. diminuta* and *S. obvelata* were collected from freshly necropsied rats and mice. After washing in phosphate-buffered saline (PBS), the specimens were maintained in small petridishes containing PBS at 37 ± 1 °C inside the incubator. Extract was tested at 10, 20 and 30 mg/ml concentrations. The reference drugs, PZQ and ABZ were tested at 1 mg/ml and 5 mg/ml

concentrations, respectively. An additional set of worms placed in PBS, served as control. All experiments were undertaken in triplicates, with five worms of each parasite in each petridish. The efficacy of extract was judged on the basis of physical motility of worms, as evident by their paralysis and mortality (Vijaya and Yadav 2016).

In vivo tests

The in vivo testing protocols in *H. diminuta*-rat model have been described previously (Yadav and Tangpu 2012). For *S. obvelata*, mice were kept in infected bed for 2 weeks. Later, establishment of infection was confirmed by cellophane tape test. Animals were divided into five groups, with 5 animals in each. Group 1 served as negative control. Group 2, 3 and 4 of animals were treated with 100 mg/kg, 200 mg/kg and 400 mg/kg doses of extract. Group 5 of mice served as positive control and received 20 mg/kg of ABZ. Eggs per gram (EPG) count of animals was done for 3 days, prior to and after dosing of extract. Treatment with extract was done for 5 days. On day 12, all the mice were sacrificed and the percentage reductions in EPG and worm counts were undertaken as described by Kozan et al. (2006), with minor modifications.

Statistical analysis

All data are presented as mean ± standard error of the mean (S.E.M). Origin Pro version 8.0 SR6 was used for graphical representation. Data was analysed using unpaired Student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey test with $p < 0.001$ being considered statistically significant.

Results

Phytochemical tests

The extract showed the presence of several plant secondary metabolites such as alkaloids, flavonoids, terpenoids, tannins, phlobatannins, saponins and glycosides (Table 1).

In vitro tests

The efficacy of the extract was observed to be dose-dependent against the test parasites. At its highest dose (30 mg/ml), the extract showed mortality of *H. diminuta* at 0.81 ± 0.01 h, as compared to PZQ with a mortality time at 5.89 ± 0.02 h. Control worms survived till 41.92 ± 0.02 h. Likewise, at the highest dose, extract revealed mortality of *S. obveleta* at 15.17 ± 0.05, as compared to 7.15 ± 0.02 h by reference drug, ABZ (Fig. 2). Control worms survived till 30.07 ± 0.09 h. The plant showed a better in vitro efficacy against *H. diminuta* compared to *S. obvelata* indicating that the plant possesses a better cestocidal activity.

In vivo tests

Administration of *S. sesban* var. *bicolor* leaf extract to rats infected with *H. diminuta* worms showed a significant reduction ($p < 0.001$) in EPG counts and worm recovery rate in a dose-dependent manner. The animals treated with 400 mg/kg dose of extract, for 5 days, showed 65.10% reduction in EPG counts, compared to the EPG counts of pre-treatment period. The reduction in worm count was found to be 56%. The animals treated with reference drug PZQ at a concentration of 5 mg/kg b.w. showed 86.62% and 76% reduction in EPG and worm counts, respectively. The EPG count in control group did not show much variation during pre-and-post treatment periods (Table 2).

The plant extract showed comparatively less activity in *S. obvelata*-mice model. During the first 3 days (pre-treatment EPG), eggs were detected in the cellophane test of all the animals. However, after 5 days treatment, EPG and worm counts reduced by 34.32% and 47.08%, respectively. ABZ (20 mg/kg), however, showed 84.72% and 93.04% reduction in EPG and worm counts, respectively (Table 3). The in vivo results also confirm that the plant possess a greater cestocidal activity than nematicidal activity.

In vitro testing of solvent phases and column fractions

Maximum in vitro efficacy was observed in the ethyl acetate phase against *H. diminuta*, which showed worm mortality at 10.17 ± 0.06 h, followed closely by chloroform phase which showed mortality at 10.76 ± 0.02, n-butanol phase at 21.59 ± 0.09 h, methanol phase at 21.84 ± 0.06 h and hexane phase at 27.46 ± 0.25 h in decreasing order of efficacy. Control worms showed physical activity till 39.37 ± 0.28 h (Fig. 3a).

Maximum in vitro efficacy was observed in the ethyl acetate phase against *S. obvelata*, which caused worm mortality at 20.70 ± 0.17 h, followed closely by chloroform phase which caused mortality at 21.12 ± 0.16 h, n-butanol phase at 21.59 ± 0.09 h, methanol phase at 21.84 ± 0.06 h and finally hexane phase at 27.46 ± 0.25 h in decreasing order of efficacy. Control worms showed physical activity till 29.63 ± 0.16 h (Fig. 3b).

The ethyl acetate phase being the most active solvent phase, was subjected to column chromatography using varying concentrations of hexane:ethyl acetate. The fractions were subjected to in vitro tests at 100 µg/ml

Table 1 Phytochemical analysis of *S. sesban* var. *bicolor* leaf extract

Secondary metabolite	Result
Alkaloid	+
Glycoside	+
Reducing sugar	–
Flavonoid	+
Terpenoid	+
Tannin	+
Phlobatannin	+
Saponin	+
Anthraquinone	–
Steroid	–

concentration to find the active fraction. Maximum in vitro efficacy was observed in 7:3 hexane:ethyl acetate, where *H. diminuta* and *S. obvelata* showed mortality at 3.56 ± 0.12 h and 9.21 ± 0.02 h respectively (Fig. 4), indicating that this fraction possesses the active principle.

Discussion

Sesbania sesban var. *bicolor* has not yet undergone proper validation to support its traditional anthelmintic claims. In vitro results of the present study revealed that at the highest concentration, maximum efficacy was observed against *H. diminuta* as compared to *S. obvelata* in a dose-

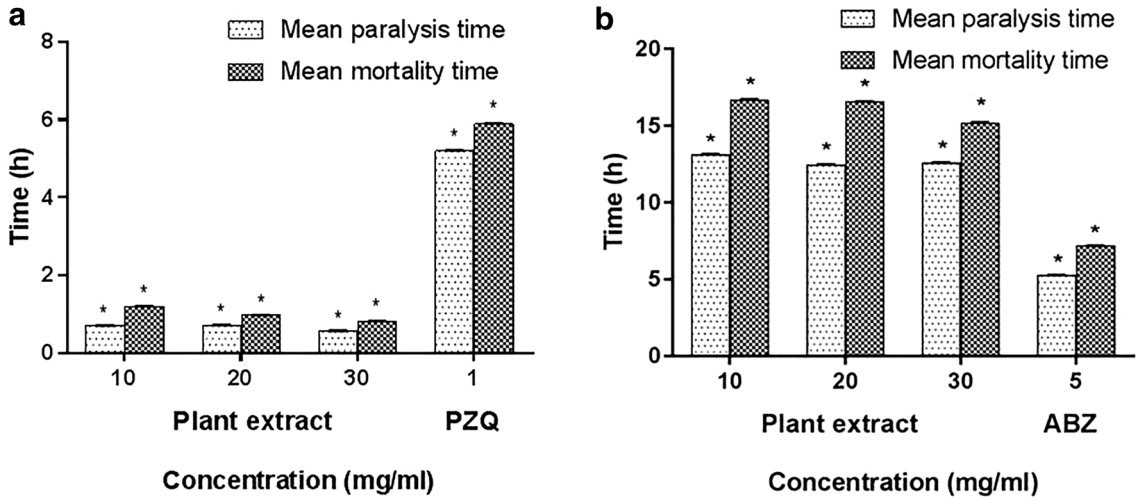

Fig. 2 In vitro anthelmintic efficacy of *S. sesban* var. *bicolor* against **a** *H. diminuta* **b** *S. obvelata*. Data is expressed as mean ± SEM. *$p < 0.0001$ compared with control group, student's t-test

Table 2 In vivo anthelmintic effect of *S. sesban* var. *bicolor* leaf extract* on *H. diminuta* infection in rats (n = 5)

Treatment groups (mg/kg × dose × day)	EPG (mean ± SEM)		Percentage difference in EPG (A − B)	Worm count at necropsy (mean ± SEM)	Percentage reduction in worm count
	Pre-treatment days 18–20 (A)	Post treatment days 26–28 (B)			
Control	21,290 ± 158	21,318 ± 168	0.13	5.0 ± 0.00	0
Plant extract					
100 × 1 × 5	21,713 ± 111	12,528 ± 123[b]	− 42.30	4.0 ± 0.31	20
200 × 1 × 5	21,461 ± 118	10,469 ± 105[b]	− 51.21	3.2 ± 0.20[a]	36
400 × 1 × 5	21,534 ± 114	7514 ± 106[b]	− 65.10	2.2 ± 0.20[a]	56
Praziquantel					
5 × 1 × 5	22,610 ± 197.57[a]	3023 ± 39.47[ab]	− 86.62	1.2 ± 0.20[a]	76

*Administration of plant extract and praziquantel on days 21–25 post inoculation with five cysticercoids/rat

[a]$p < 0.001$ as compared to control value, [b]$p < 0.001$ as compared to pre-treatment, one-way ANOVA, followed by Tukey's test

Table 3 In vivo anthelmintic effect of *S. Sesban* var. *bicolor* leaf extract* on *S. obvelata* in mice (n = 5)

Treatment groups (mg/kg × day × dose)	EPG (mean ± SEM)		Percentage difference in EPG	Worm count at necropsy		Percentage reduction in worm count
	Pre-treatment (days 1–3)	Post treatment (days 9–11)		Min–Max	Mean ± SEM	
Control	29 ± 0.89	29.8 ± 0.66	2.6	47–120	89.2 ± 15.24	0
Plant extract						
100 × 1 × 5	27.8 ± 1.15	21.2 ± 1.31	− 23.74	48–98	65 ± 8.84	27.13
200 × 1 × 5	25.6 ± 1.80	17.2 ± 0.86	− 32.81	33–83	53.4 ± 9.41	40.13
400 × 1 × 5	26.8 ± 1.46	17.6 ± 1.96[b]	− 34.32	28–81	47.2 ± 10.02	47.08
Albendazole						
20 × 1 × 5	28.8 ± 1.15	4.4 ± 1.02[b]	− 84.72	5–8	6.2 ± 0.58[a]	93.04

*Administration of plant extract and albendazole on days 4–8 after pre-treatment EPG

[a]$p < 0.001$ as compared to control value, [b]$p < 0.001$ as compared to pre-treatment, one-way ANOVA, followed by Tukey's test

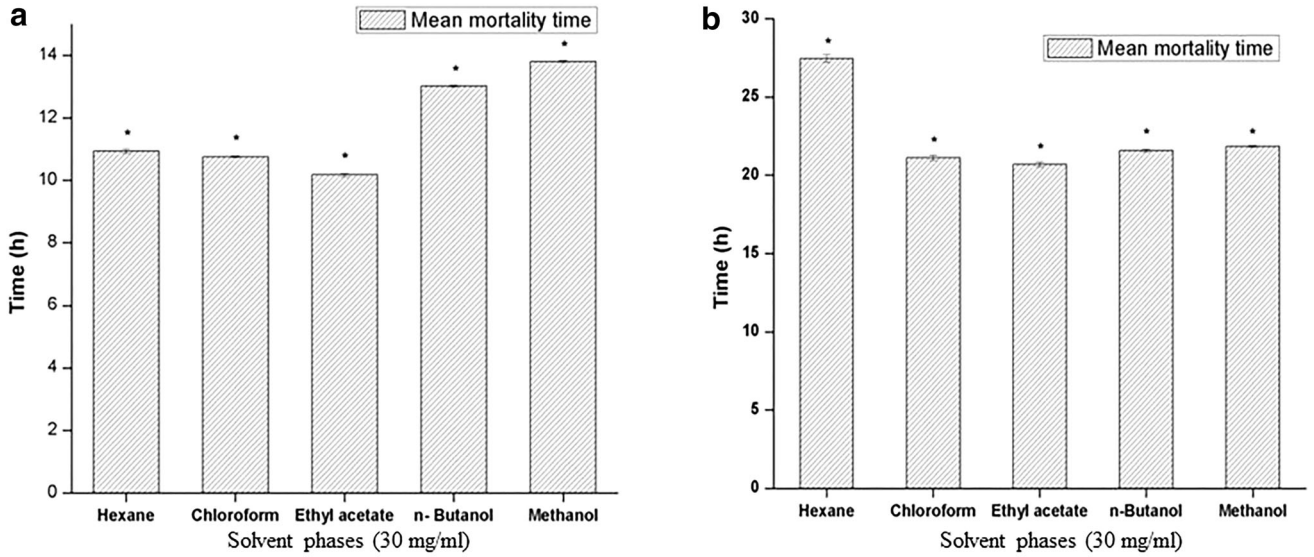

Fig. 3 In vitro anthelmintic effects of different solvent phases obtained from *S. sesban* var. *bicolor* methanolic crude extract against **a** *H. diminuta*, **b** *S. obvelata*. Data is expressed as mean ± SEM. *$p < 0.001$ compared with control group, student's t-test

dependent manner, indicating that the plant possesses a better cestocidal activity. These findings are in agreement with the reports of other workers who studied the anthelmintic potentials of medicinal plants. In vitro studies of *S. sesban* have shown its hydroethanolic and aqueous leaf extract to be effective against cestode, *Moneizia expansa* and *Paramphistomum* flukes (Limsay et al. 2014). Tandon et al. (1996) reported that the root tuber peel extract of *Flemingia vestita,* when tested against commonly available helminth parasites such as, *Ascaris suum, A. lumbricoides, Heterakis gallinarum, Raillietina echinobothrida, Paramphistomum* sp., showed in vitro anthelmintic efficacy. An in vitro anthelmintic assay of *Alpinia nigra* showed that the ethanolic extract possessed effective anthelmintic efficacy against *Fasciolopsis buski* (Swargiary and Roy 2015). In a related study by Bøgh et al. (1996), on anthelmintic efficacy of the dried fruits of *Embelia schimperi*, the plant extract showed significant in vitro as well as in vivo effects against *H. diminuta*. However, there was no in vivo effect against *Hymenolepis microstoma*, *Echinostoma caproni* and *Heligmosomoides polygyrus*, although there was in vitro efficacy. Thus, the in vitro study indicates that *S. sesban* var. *bicolor* possess anthelmintic property and to substantiate the present findings, further study was undertaken to test the extract in two in vivo models.

In vivo assay revealed that at the highest dose, rats infected with *H. diminuta,* showed a higher reduction of worm and EPG counts as compared to mice infected with *S. obvelata.* In a similar study by Nath and Yadav (2015), the leaf extract of *Hibiscus rosa-sinensis* showed promising

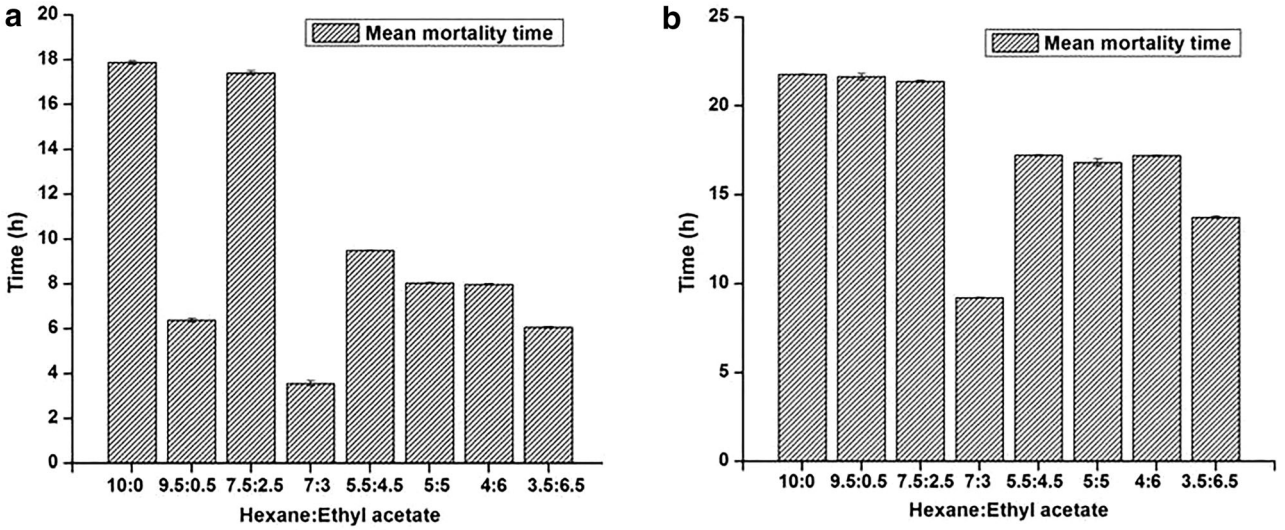

Fig. 4 In vitro anthelmintic effects of column fractions obtained from ethyl acetate phase of *S. sesban* var. *bicolor* methanolic crude extract against **a** *H. diminuta*, **b** *S. obvelata*. Data is expressed as mean ± SEM. $^{*}p < 0.001$ compared with control group, student's t-test

anticestodal efficacy. Interestingly in a similar study by Gogoi and Yadav (2016), the methanolic extract of *Caesalpinia bonducella* showed a better in vivo nematicidal efficacy. In another report by Sapaat et al. (2012), papaya seeds were tested for anthelmintic efficacy against *H. diminuta* and there was a reduction in EPG as well as worm counts in a dose-dependent manner. This may be due to the various phytochemicals present in each of these plant parts which bring about such an effect. In a study by Vijaya et al. (2018) on in vitro and in vivo anthelmintic efficacy of two phytochemicals, ursolic acid and betulinic acid against *S. obvelata*, the result showed a dose-dependent efficacy. Therefore it may be stated that phytochemicals play an active role in a plant's efficacy and hence, phytochemical tests on *S. sesban* var. *bicolor* were performed.

Phytochemical tests revealed the presence of alkaloids, glycosides, flavanoids, terpenoids, tannins, phlobatannins and saponins. Other studies, however, did not show the presence of saponin (Mythili and Ravindhran 2012). The presence of alkaloids, flavonoids, terpenoids, tannins, phlobatannins, saponins and glycosides has also been reported in a study on the phytochemistry of the genus *S. sesban* by Kumar et al. (2014). According to Bauri et al. (2015), phenolic compounds, flavonoids and tannins interfere with the energy generation mechanism and/or the glycoprotein of the cell surface/cuticle of parasites leading to their death. Likewise, alkaloids have also been reported to act on the central nervous system of parasites and lead to their paralysis. The active column fraction can be further studied to find the active compound followed by its characterization. This active compound can further undergo preclinical tests before it can be applied to human use.

In conclusion, the findings of the present study show that *S. sesban* var. *bicolor* leaf extract possesses significant anthelmintic anticestodal efficacy although the effect was comparatively less in nematode, and validates its traditional claims as an anthelmintic among the Santhal tribe. Therefore, the findings of this study bear relevance with regard to the fact that a large majority of the people in this region consume pork and beef in their staple diets. The consumption of these meats poses a threat for cestode infection in this region. Thus the traditional use of this plant against cestode infection will be useful in the traditional medicine of the Santhal tribe.

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Authors' contributions All the authors have contributed equally to this study.

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Availability of data and material All data generated or analysed during this study are included in this published article [and its supplementary information files].

Compliance with ethical standards

Conflict of interest All the authors declare that they have no conflict of interest.

Ethical approval All experiments on rats and mice were done after due approval from the Institutional Animal Ethics Committee (Animal Models) of North-Eastern Hill University, Shillong (Vide, Member Secretary, IEC, NEHU, dated December 4, 2014).

Consent to participate Not applicable.

Consent for publication Not applicable.

Code availability All data and materials as well as software application or custom code support their published claims and comply with field standards.

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