



GC-MS, antioxidant study and effect of *Sesbania sesban* var. *bicolor* on *Raillietina echinobothrida* and *Syphacia obvelata*

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Received: 22 July 2023 / Accepted: 4 April 2024 / Published online: 25 April 2024

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Abstract

Anthelmintic medicinal plants can cause deformities in the external structure of parasites exposed to them. *Sesbania sesban* var. *bicolor* is a traditionally used anthelmintic medicinal plants used by the Santhal tribe of Assam. This study evaluates the damages caused by the methanolic extract on the tegument of *Raillietina echinobothrida* and *Syphacia obvelata* and also assesses its antioxidant activity. In addition, GC-MS study to identify the phytochemicals in the studied plant was also carried out. Worms were exposed to 30 mg/ml of the extract and the paralysed worms were processed for scanning electron microscopy study. Images obtained revealed extensive damage on the mouth, suckers, and cuticle/tegument of the worms. The study revealed that the plant possesses a good antioxidant activity. GC-MS of the plant revealed the presence of about 21 prominent peaks of which Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester was the most abundant. The activity of the plant may be attributed to the presence of these compounds which may be responsible for its antioxidant and anthelmintic activity.

Keywords Anthelmintic · Antioxidant · *Raillietina echinobothrida* · *Sesbania sesban* var. *bicolor* · *Syphacia obvelata*

Introduction

The prevalence of helminthiasis in rural areas of developing countries is alarming (Mkhize-Kwitshana et al. 2022). About two billion which is almost a quarter of the world's population are known to harbour helminth worms (Al Amin and Wadhwa 2022). The risk factors of helminthiasis include poor sanitation, bad economic status, poor hygiene, lack of clean drinking water, lack of health facilities, and improper dwelling conditions (Al Amin and Wadhwa 2022). Communities residing in these countries are known to use their own traditional medicines consisting of medicinal herbs to cure various ailments including helminth infestations. They are also a component of their primary health care (Deli et al. 2022). About one-third of the world's

population lack access to quality health care and in such areas, traditional remedies have become the sole mode of treatment (WHO 2022). Several traditionally used medicinal plants have studied and found to be potent anthelmintics such as *Digitaria eriantha* (Castañeda-Ramírez et al. 2022), *Lepidagathis hyalina* (Islam et al. 2022), and *Isatis tinctoria* (Ragusa et al. 2022). The effects of medicinal plants on the worms have been studied by scanning electron microscopy (SEM) studies where the tegument can be observed for any deformities (ElGhannam et al. 2023; El-Wakil et al. 2023).

Sesbania sesban var. *bicolor* (Fabaceae) is a medicinal plant used to treat helminthiasis by the Santhals of Assam in India. It has been shown to possess both in vitro and in vivo efficacy against cestode, trematode and nematode models (Soren et al. 2021). The effect of these plants in the tegument of *Gastrothylax crumenifer* (Creplin, 1847) (Trematoda) have already been studied (Soren and Yadav 2021). Several authors have used *Raillietina echinobothrida* and *Syphacia obvelata* as model parasites to evaluate the effects of plant extract on their tegument (Roy et al. 2012; Dasgupta et al. 2013; Vijaya et al. 2018). This study evaluates the effects caused by the methanolic plant extracts of *Sesbania sesban* var. *bicolor* (leaves) on the tegument of fowl

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tapeworm *R. echinobothrida* Megnin, 1880 (Cestoda) and mice pinworm *S. obvelata* Rudolphi, 1802 (Nematoda).

Medicinal plants are also known to possess anti-oxidant properties. Progressive damage of DNA, lipids, proteins, and other essential macromolecules is caused by free radicals, such as reactive oxygen and nitrogen species, which are byproducts of normal physiological metabolism. A number of cardiovascular, neurological, cancer, and even aging-related clinical health issues are caused by the effect known as oxidative stress. Moreover, reactive oxygen species (ROS) are oxygen radicals that control important transcription factors that affect cell signaling pathways involved in growth, differentiation, and apoptosis. Thus, oxidative stress can change a number of crucial processes that have an impact on embryonic development both favorably and unfavorably (Dennerly 2007). Exogenous antioxidants, which are mostly found in plants, are essential for slowing down or stopping the oxidation process by either eradicating free radicals or converting them into harmless metabolites. Because of this, dietary antioxidants are the main line of defense against cellular oxidation.

Plants belonging to Fabaceae family are the second largest family of medicinal plants comprising more than 490 species. They are highly acclaimed and valued for their historic use in treating a variety of illnesses such as for treatment of anemia, menorrhagia during pregnancy, ulcers, polymenorrhea, diarrhoea, spleen enlargement, diabetes, hypocholesterolaemic conditions, Kwashiorkor, and thyroxine-induced hyperglycaemia. Their use as anthelmintic and as an antioxidant, anti-inflammatory is quiet notable among tribal communities (Goswami et al. 2016). Therefore, the medicinal benefits of the plant, such as its anti-inflammatory, anti-oxidant, and anthelmintic properties, must thus be studied.

Materials and methods

Collection of parasites and SEM study

As described in an earlier study, plant material was collected from local habitats and extracted in methanol (Soren et al. 2021). *Raillietina echinobothrida* was collected from freshly necropsied fowl intestine from local markets whereas *Syphacia obvelata* was collected from mouse intestine maintained in the laboratory. Collected worms were placed in phosphate buffered saline (PBS) for further evaluations. Worms ($n=5$) were placed in 30 mg/ml concentration of the plant extract in triplicates. On paralysis, worms were washed in neutral buffered formalin (NBF) thrice, dehydrated in acetone grades and dried using tetramethylsilane (TMS). These worms were then viewed in a Hitachi

TM 4000 Plus SEM. The results were compared with control worms placed in PBS and reference drug praziquantel (PZQ) for cestode and albendazole (ABZ) for nematode worms (Soren et al. 2021).

Antioxidant activity

Determination of diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

DPPH stable free radical scavenging activity was determined by following the standard protocol (Blois 1958). 1 ml of 0.1 mM of DPPH prepared in methanol was added to 3 ml of different concentrations (10, 20, 40, 60, 80 & 100 μg) of plant extracts and incubated at 37 °C for 30 min. BHT (Butylated hydroxytoluene) was used as a standard reference. Absorbance was measured at 517 nm against control using double beam UV-Vis Spectrophotometer (LABTRONICS LT 2700). The percentage of inhibition was calculated by comparing the absorbance values of the test samples with those of the control. The inhibition percentage (I) was calculated as follows:

$$\text{Inhibition \%} = \left\{ \frac{\text{Absorbance of control} - \text{Absorbance of sample}}{\text{Absorbance of control}} \right\} \times 100.$$

Potassium ferricyanide scavenging assay

Reducing power was determined according to the method described by Oyaizu (1986) using ascorbic acid as standard. 1 ml of the extract and 1 ml of the standard with various concentrations (10, 20, 40, 60, 80, and 100 $\mu\text{g}/\text{ml}$) were mixed with 2.5 ml of phosphate buffer (6.6 pH) and 2.5 ml of 1% potassium ferricyanide. The mixture was then incubated at 50 °C for 30 min. The reaction was stopped by adding 2.5 ml of 10% trichloroacetic acid and the mixture was centrifuged at 3000 rpm for 10 min. 2.5 ml of the supernatant was mixed with 2.5 ml of distilled water, and 0.5 ml of 0.1% ferric chloride solution and the absorbance was taken at 700 nm using UV-Vis spectrophotometer.

Total phenol content

The total phenolic content of the plant was determined by using the method of McDonald et al. (2001) with slight modifications. Calibration curve was prepared by mixing 1 ml of methanolic solution of gallic acid (10, 20, 40, 60, 80, and 100 $\mu\text{g}/\text{ml}$) with 5 ml Folin-Ciocalteu reagent (which was diluted tenfold from the original stock). After 3 min, 4 ml of sodium carbonate solution (0.7 M) was added, and the mixture was allowed to stand for 1 h at room temperature. Absorbance was measured at 765 nm using UV-Vis

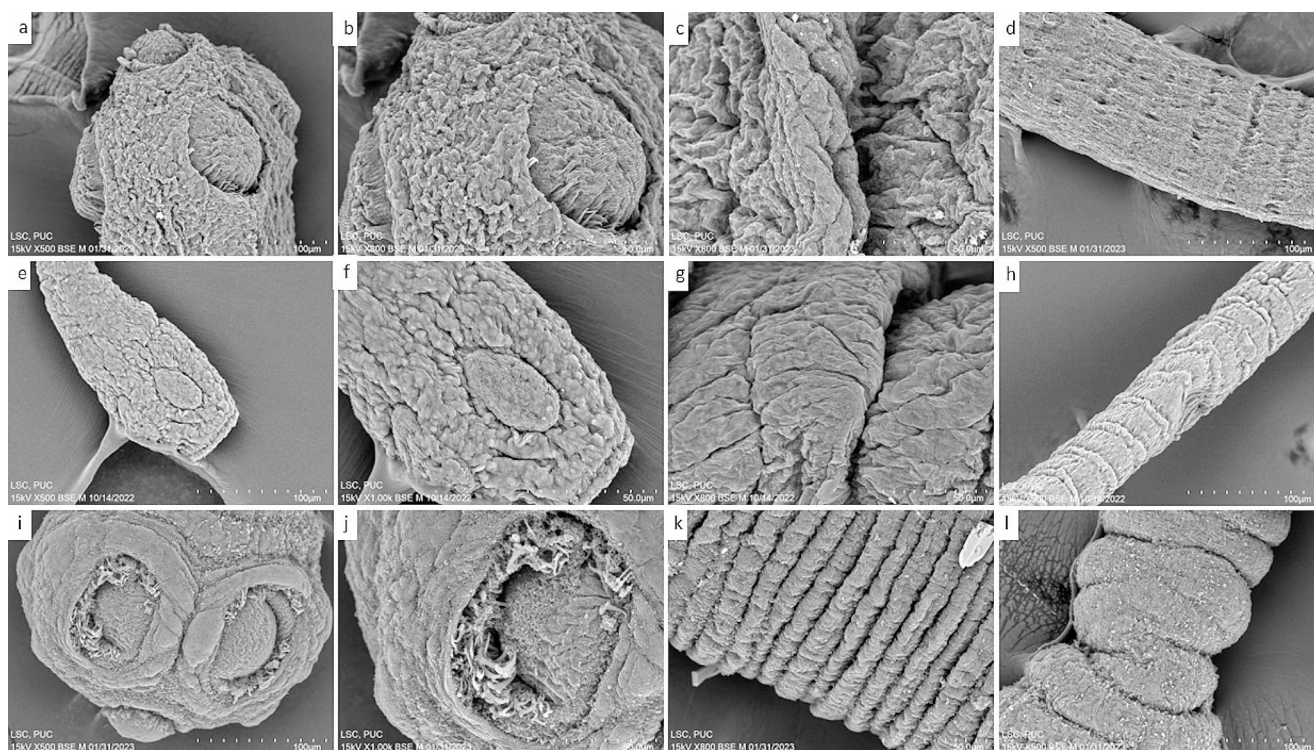


Fig. 1 *Raillietina echinobothrida*. **a** Scolex of control worm, **b** sucker and microtriches of control worm, **c** tegument of control worm, **d** segments of control worm, **e** scolex of extract treated worms, **f** sucker and microtriches of extract treated worm, **g** tegument of extract treated

worm, **h** segments of extract treated worm, **i** scolex of PZQ treated worms, **j** scolex and microtriches of PZQ treated worms, **k** tegument of PZQ treated worms, **l** segments of PZQ treated worms

spectrophotometer (LABTRONICS LT 2700). 1 ml extract (50 µg/ml) was also mixed with the reagents above and after 1 h interval, absorbance was measured to determine total plant phenolic content. From the calibration curve, the content of phenolic compounds was determined and expressed as milligrams of Gallic acid equivalent (GAE)/g of the dried extract.

Total flavonoid content

The total flavonoid content was determined by the aluminium chloride method. 1 ml of the extract (50 µg/ml) was mixed with 2 ml of distilled water. After 5 min, 3 ml of 5% sodium nitrite (NaNO₂) and 0.3 ml of 10% aluminium chloride (AlCl₃) were added. After 6 min, 2 ml of NaOH (1 M) was added, and the volume was made up to 10 ml with distilled water. After 1 h, absorbance was taken at 510 nm in a UV-Vis spectrophotometer (LABTRONICS LT 2700). Standard curve was prepared with quercetin at different concentrations (10, 20, 40, 60, 80, and 100 µg/ml). From the calibration curve of the reference standard, the total flavonoid content was determined and expressed as milligrams of quercetin equivalent (QE/g) of dried extract.

Total antioxidant activity

The total antioxidant activity of extract was determined by phosphomolybdate estimation using ascorbic acid as a standard. 0.1 ml of sample (100 µg) solution was mixed with 1 ml of reagent solution containing 0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate. Incubation was done at 95 °C for 90 min after which they were cooled and absorbance was measured at 695 nm. The total antioxidant activity was measured as ascorbic acid equivalent of the dried plant extract.

Gas chromatography-mass spectrometry (GC-MS)

Sesbania sesban methanol extract was analysed in a single quadrupole GC-MS system (Thermo Scientific TRACE™ 1300 ISQ™ LT). The plant extract was dissolved in acetonitrile in a ratio of 50 mg in 3 ml. A nonpolar column TR-5MS (260F142P) was used as a stationary phase. The dimension of the column was 30 m × 0.25 mm × 0.25 µm with film thickness of 0.25 µm. The injector port was set at a temperature of 250 °C. The oven temperature was initially set at 70 °C for 2 min and incrementally raised by 10 °C up to 250 °C. Helium was used as a carrier gas and was released into the oven chamber at a constant flow rate of 1 ml/min.

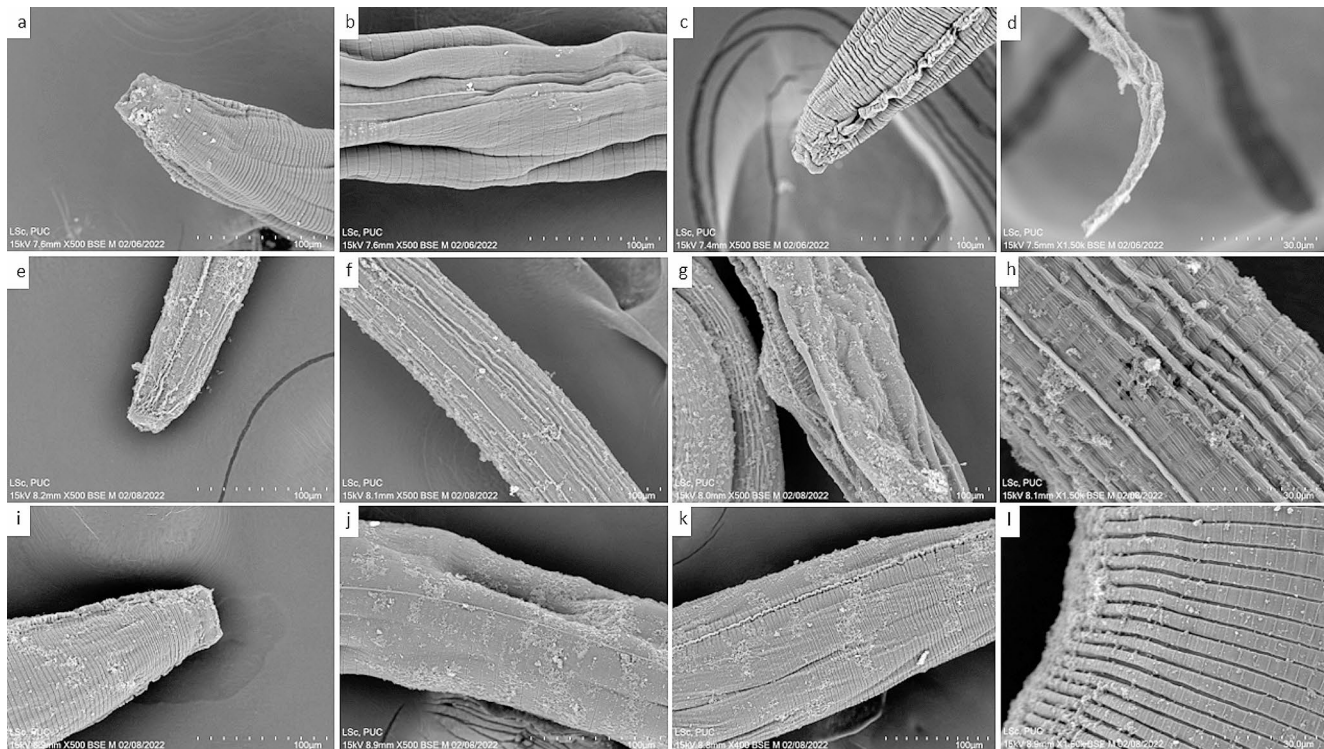


Fig. 2 *Syphacia obvelata*. **a** Mouth region of control worm, **b** cuticle of control worm, **c** annuli of control worm, **d** tail region of control worm, **e** mouth region of extract treated worm, **f** cuticle of extract treated worms, **g** deformed annuli of extract treated worm, **h** annulations of

the cuticle of extract treated worm, **i** mouth region of ABZ treated worm, **j** cuticle of ABZ treated worm, **k** cuticle showing annulations of ABZ treated worm, **l** annuli of ABZ treated worm

Sample injection was done in a volume of 1 μ l in split mode and the splitting ratio was maintained at 1:50. The mass spectrometer was run with ionization electron energy of 70 eV. Ion source and transfer line temperature were set at 250 $^{\circ}$ C. The total running duration was 55 min. Mass ratio (m/z) was scanned up to 1100 Da. The final chromatogram was generated with Thermo Scientific™ Xcalibur™ software. Compounds were identified on the basis of their retention time, chemical formula, and molecular weight from libraries of Wiley Registry™ (10) and National Institute of Standards and Technology database.

Results

SEM study

Control *R. echinobothrida* worms showed normal architecture with healthy scolex having opened suckers, intact hooks and healthy cuticle. Segments did not appear shrunken. Worms exposed to plant extract showed deformities in the form of clogged suckers, completely damaged hooks, and a cuticle that appeared shrunken. Also, the segments had shriveled in reaction to the plant extract. Worm exposed to PZQ also showed extensive deformities where

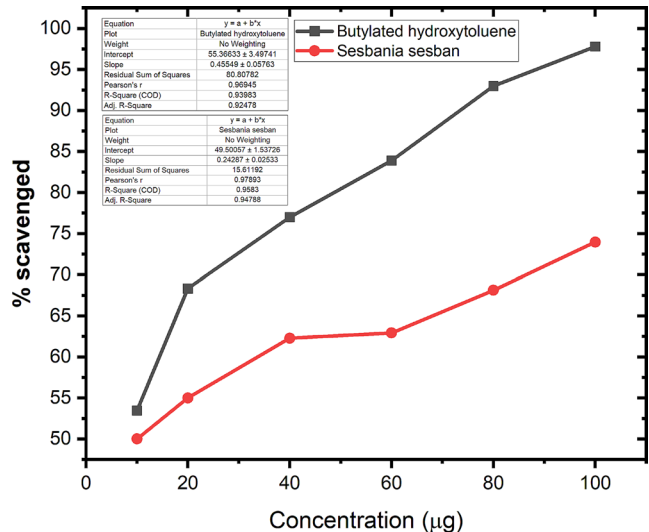


Fig. 3 DPPH radical scavenging assay using BHT as standard

segments had compressed, scolex appeared shrunken, and hooks appeared partially damaged (Fig. 1).

Control *S. obvelata* worms showed normal architecture with an open mouth and striations in the tegument were found to be uniformly arranged. Worms exposed to plant extracts showed wrinkled tegument, and the transverse annulations were distorted. Also, the segmentations were

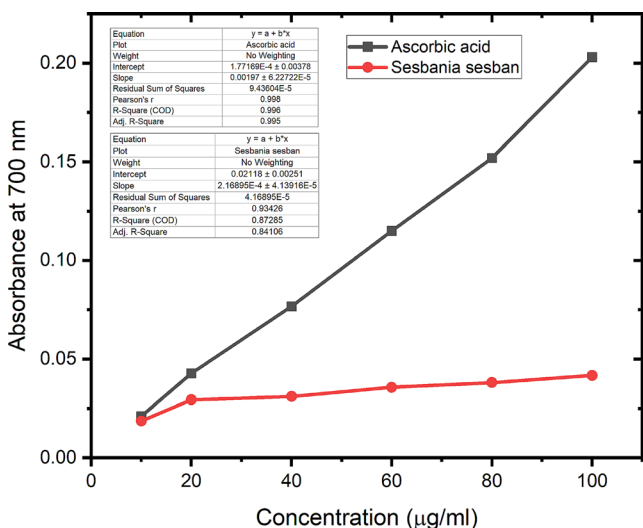


Fig. 4 Potassium ferricyanide scavenging assay using ascorbic acid as standard

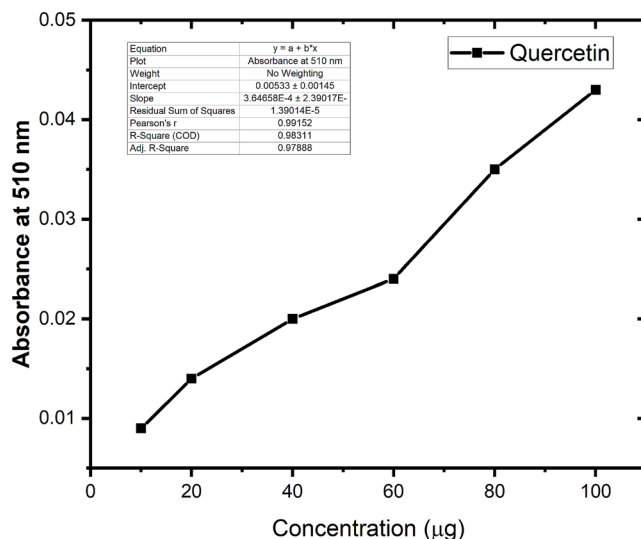


Fig. 6 Total flavonoid content using quercetin as standard

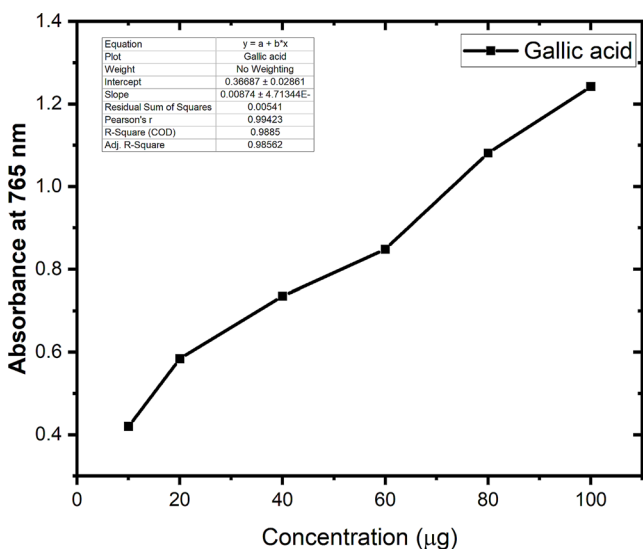


Fig. 5 Total phenol content using gallic acid as standard

found to be distorted. However, worms exposed to ABZ showed mild deformities. Segments appeared to be less distorted than the extract treated worms and appeared to be uniformly arranged (Fig. 2).

Antioxidant assay

DPPH radical scavenging assay measurements of the free radical scavenging activities revealed that both the extract and standard BHT showed concentration-dependent increase in scavenging activities. At all concentrations, the extract activities were, however, somewhat below the norm indicating that *S. sesban* is a promising source of natural antioxidants in scavenging harmful free radicals (Fig. 3). The extract activities in scavenging potassium ferricyanide

showed concentration-dependent increase in activities, although they were still slightly inferior to the standard ascorbic acid at various doses (Fig. 4). Phenolic compounds are one of the main groups of antioxidants found in plants that serve as a depiction for plant’s phenol content overall. Reaction of gallic acid with Folin-Ciocalteu reagent was used to determine the total phenolic concentration equivalent of the acid. It was found that 1 gram of the extract contains 3.345 mg of gallic acid equivalent dry weight of the plant extract (Fig. 5). Flavonoids are another important class of antioxidants found in plants. The total flavonoid content of the extract was measured upon reactions with aluminum chloride, sodium nitrite and sodium hydroxide. The reaction product shows that 1 gram of extract contains 59.56 mg of quercetin equivalent dry weight of the sample (Fig. 6). The total antioxidant activity was estimated upon

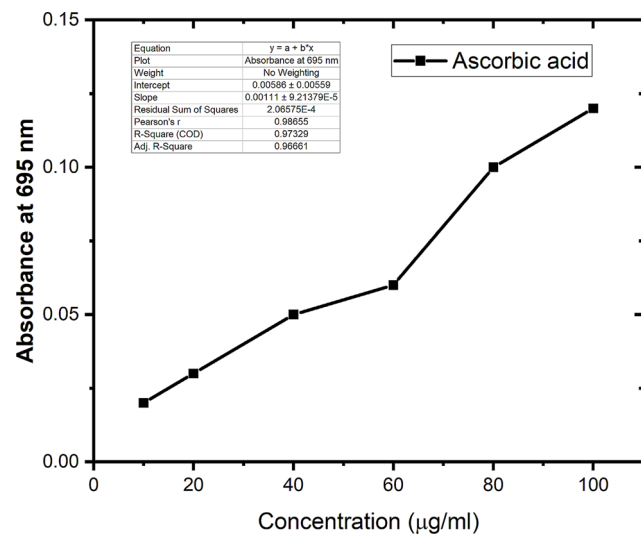


Fig. 7 Total antioxidant activity using ascorbic acid as standard

reaction with ammonium molybdate, sodium phosphate and sulphuric acid. The end product of the reaction shows that 1 gram of the extract contains 63.93 mg of ascorbic acid equivalent dry weight of the plant extract (Fig. 7).

Gas chromatography-mass spectrometry (GC-MS)

The chemical constituents of *S. sesban* extract analyzed from GC-MS (Thermo Scientific TRACE™ 1300 ISQ™ LT, USA) showed 21 prominent peaks (Fig. 8). The detected compounds are described in Table 1. The most abundant compound was octadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester, which was detected at retention time 25.87 with relative abundance of 76.34. Other major compounds detected were n-hexadecanoic acid (retention time 13.43, relative abundance 70.77), phytol (retention time 16.21, relative abundance 71.56), hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl) ethyl ester (retention time 22.79, relative abundance 74.72), and bufa-20,22-dienolide, 3,14-dihydroxy-, (3 α ,5 α)- (retention time 31.14, relative abundance 74.70).

Discussion

SEM studies are routine studies carried out by several workers to study the effect of extracts or drugs on parasites surface. This validates the extent of damage an extract or drug can cause. The cuticle or tegument plays an important role in protection and absorption and hence its damage can cause death of the worm (Lalchandama et al. 2009). There are reports of in vitro anthelmintic efficacy by several authors against these two studied worms. However, these studies fail to produce SEM studies of the body surface (Gogoi and Yadav 2016; Nath and Yadav 2016). This plant has been studied for its in vitro efficacy and results show that it possesses promising anthelmintic efficacy against *G. crumenifer*, *Hymenolepis diminuta* (Rudolphi, 1819) and *S. obvelata* (Soren and Yadav 2021; Soren et al. 2021). This study evaluates the damage caused by the extracts on their body surface.

Raillietina echinobothrida has been used as a model by various workers to study the effects caused by extracts. In a similar study, Dasgupta et al. (2010) observed vacuolization and erosion of microtriches on the tegument of worms exposed to *Acacia oxyphylla*. Likewise, Roy et al. (2012) also showed that worms exposed to a compound from *A.*

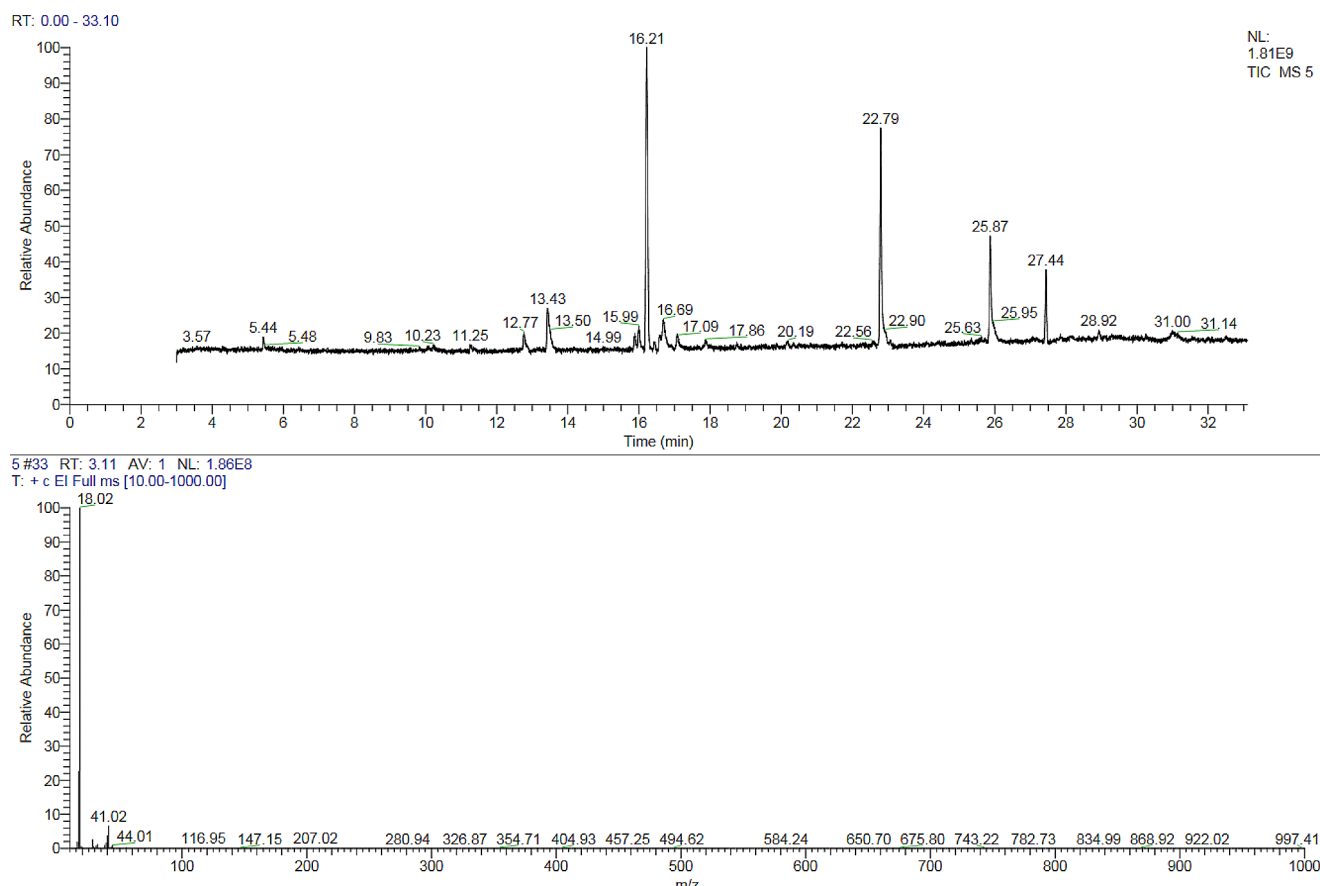


Fig. 8 GC-MS of *S. sesban* showing 21 prominent peaks

oxyphylla caused extensive erosion of the tegument. Dasgupta et al. (2013) carried out studies using *Securinega virosa* extract against *R. echinobothrida*. They observed destruction of the tegument with swellings and vacuolization. Deori and Yadav (2016) also carried out SEM studies against rat tapeworm *H. diminuta* and showed that the plant extract caused damages to the scolex, tegument and microtriches. Likewise, this study also revealed considerable damage in the tegument of the worms.

Vijaya et al. (2018) had earlier studied the effect of plant extracts on the architecture of *S. obvelata*. They observed distortions in the cuticular annulations as observed in this study as well. Most extracts are known to act on the parasites via a tegumental mode (Soren et al. 2021). Lalchandama et al. (2009) also carried out a similar study against *Ascaridia galli* Schrank, 1788 (nematode) and found similar results where the tegument was found to be extensively damaged on exposure to *A. oxyphylla* stem bark extract. Such damaged body surface will not be able to function as a defensive barrier thereby annihilating the worm. SEM studies on *S. obvelata* are however scanty.

Bioactive chemicals found in *S. sesban* have been reported and such includes phenols, flavonoids, anthocyanins, proteins, phytosterol, gums, campesterol, delphinidin glucosides, beta-sitosterol, cyanidin, alpha-ketoglutaric, pyruvic acid, oxaloacetic acid, alkaloids, and fixed oils. These phytochemical analyses led to the isolation of

3-β-D-glucuronide, galactomannan, oleanolic acid, and stigmastane-5.24(28)-diene-3β-O-β-D-galactopyranoside from the plant. Numerous biological processes, including antioxidant, central nervous system stimulant, anti-diabetic, anti-microbial, and anti-inflammatory properties, are believed to be mediated by these compounds. Additionally, *S. sesban* has also been reported for its use in the treatment of schistosomiasis and as mosquito repellent (Gomase et al. 2012; Nigussie and Alemayehu 2013). The plant is also commonly used to treat various parasitic illnesses among the Santhal tribe of Assam, Northeast India (Soren et al. 2021).

In addition to its beneficial antiparasitic characteristics, *S. sesban* is well known for its antioxidant properties. Plant-derived antioxidants neutralize free radicals by donating electrons to free radicals, which prevents it from reacting with other molecules thereby preventing damage to cells and tissues. Several studies have investigated the total phenolic content, flavonoid content, and total antioxidant activity of *S. sesban*. The DPPH free radical scavenging activities, total phenol content and total flavonoid content of *S. sesban* leaves were evaluated using a UV-vis spectrophotometer. The percentage of DPPH scavenging activity of ethanol extract at 50 µg/ml was found to be 52.87 while that of ethyl acetate extract and n-hexane extracts were 52.58 and 51.31, respectively. Total phenol content and total flavonoid content estimation similarly showed that ethanol

Table 1 Compounds detected in the GC-MS study of *S. sesban*

Sl No.	Retention time	Relative abundance	Compound	Formula	Molecular weight
1	5.44	41.10	Phenol, 2,5-bis(1,1-dimethylethyl)-	C14H22O	206
2	9.83	90.63	Water	H2O	18
3	10.23	15.79	5,9-Dimethyl-2-(1-methylethylidene)-1-cyclodecanol	C15H28O	224
4	11.25	20.53	2-Pentadecanone, 6,10,14-trimethyl-	C18H36O	268
5	12.77	25.94	Tetradecanoic acid, 12-methyl-, methyl ester, (S)-	C16H32O2	256
6	13.43	70.77	n-Hexadecanoic acid	C16H32O2	256
7	15.99	13.51	Cyclopropaneoctanoic acid, 2-[[2-[(2-ethylcyclopropyl)methyl]cyclopropyl]methyl]-, methyl ester	C22H38O2	334
8	16.21	71.56	Phytol	C20H40O	296
9	16.69	7	2-Methyl-Z, Z-3,13-octadecadienol	C19H36O	280
10	17.09	44.37	Octadecanoic acid, 2-(2-hydroxyethoxy)ethyl ester	C22H44O4	372
11	20.19	8.90	(Z)-Dodec-5-en-4-olide	C12H20O2	196
12	22.56	66.34	Water	H2O	18
13	22.79	74.72	Hexadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C19H38O4	330
14	23.05	30.62	Oxiraneundecanoic acid, 3-pentyl-, methyl ester, trans-	C19H36O3	312
15	25.63	10.05	Octadecanal, 2-bromo-	C18H35BrO	346
16	25.87	76.34	Octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester	C21H42O4	358
17	27.07	41.66	Androstane-11,17-dione, 3-[[trimethylsilyl]oxy]-, 17-[O-(phenylmethyl)oxime], (3α,5α)-	C29H43NO3Si	481
18	27.44	36.14	Squalene	C30H50	410
19	28.92	44.73	Spirost-8-en-11-one, 3-hydroxy-, (3α,5α,14α,20α,22α,25R)-	C27H40O4	428
20	31.00	24.31	Bufa-20,22-dienolide, 3,14-dihydroxy-, (3α,5α)-	C24H34O4	386
21	31.14	74.70	Bufa-20,22-dienolide, 3,14-dihydroxy-, (3α,5α)-	C24H34O4	386

extract possessed 5.18 g GAE/100 g and 3.22 g QE/100 g showing that the results are fairly comparable to the present study. Additionally, it was reported that total phenol content had significant and negative correlation with scavenging activities i.e., increasing phenolic content leads to increase in free radical scavenging activities. On the other hand, total flavonoid content was found to have no significant correlation with scavenging activities (Fitriansyah et al. 2017). According to Kathiresh et al. (2011), methanol extract of *S. sesban* flower had DPPH scavenging percentage of 37.09 in which anthocyanins are believed to be responsible for this activity. Study on the DPPH scavenging activities of dried seed extracts also showed that the extract revealed concentration dependent increase in scavenging activities. (Mani et al. 2011)

Sesbania sesban is reported to contain several bioactive compounds including oleanolic acid, galactomannan, delphinidin glucosides, 3- β -D-glucuronide, alpha-ketoglutaric, stigmastane-5,24(28)-diene-3 β -O- β -D-galactopyranoside, cyanidin, oxaloacetic, pyruvic acids, cholesterol, campesterol, and beta-sitosterol (Goswami et al. 2016). It is a rich source of vitamins, antioxidants, proteins and important essential minerals including iron, sodium, phosphorous, and potassium. Vital chemical markers such as myo-inositol, pinitol, fructose, sucrose, and α -D-glucopyranoside were also identified in the seeds (Singh et al. 2023). Phenol, 2,5-bis(1,1-dimethylethyl)-, tetradecanoic acid, 12-methyl-, methyl ester, (S)-, and n-hexadecanoic acid detected in the current investigation were similarly found in other species of *Sesbania* i.e., *S. grandiflora* L. (Hussain and Kumaresan 2014). Most abundant compound i.e., octadecanoic acid, 2-hydroxy-1-(hydroxymethyl)ethyl ester, a fatty acid ester is reported for its potent antioxidant activity. Other notable compounds such as n-hexadecanoic acid and phytol are known for their antimicrobial, antimutagenic, cytotoxic, antispasmodic, antidiabetic, anticonvulsant, antinociceptive, antidepressant, and anti-inflammatory activities (Tyagi and Agarwal 2017; Lalthanpuui et al. 2020). These phyto-compounds of *S. sesban* are the plausible active component responsible for the antioxidant and anthelmintic properties and require further rigorous investigation.

Conclusions

The study suggests that the exposure of these studied plants can cause deformities on the external body surface of parasite worms establishing a good in vitro efficacy which could be the probable reason for their mortality. According to antioxidant analyses, the plant is a good source of antioxidants that are helpful for overall health and are responsible in the fight against oxidative stress and diseases brought on by free

radicals. Further evidence that *S. sesban* is a likely source of anthelmintic chemicals comes from the specific malformations that the plant's compounds inflicted on the treated parasites.

Acknowledgements The authors thank Professor Arun K. Yadav for his guidance during the experiment period. Author also thank the Directorate of Forensic Science, Government of Mizoram, MINECO, Aizawl for the GC-MS study results.

Author contributions ADS conceptualised the study, carried out the experiments and wrote the first draft. PBL and KL performed microscopy, GC-MS and edited the draft. All authors read and approved the final draft.

Funding No funding was received from any organization or individual to carry out this work.

Data availability All data generated or analysed during this study are included in this published article.

Declarations

Ethical approval The use of mice was approved by the Institutional Animal Ethics Committee (Animal Models) of North-Eastern Hill University, Shillong (Vide, Member Secretary, IEC, NEHU, dated December 4, 2014).

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

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