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Chemical profiling of alkylamides from the "herbal Botox", *Acmella oleracea*, cultivated in Mizoram and their pharmacological potentials

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Abstract

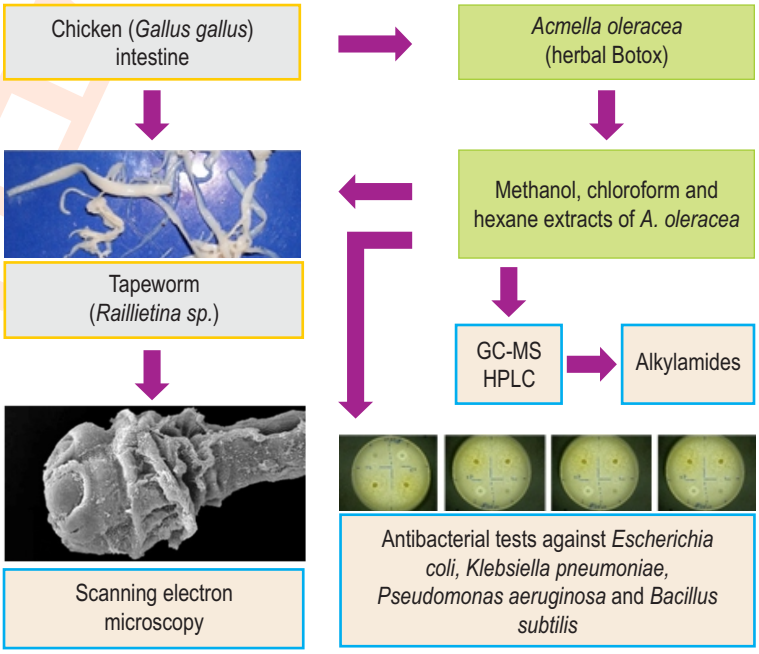
Aim: To perform chemical analysis and study the antibacterial and antiparasitic activities of *Acmella oleracea* extracts.

Methodology: The methanol, chloroform, and hexane extracts of *A. oleracea* were prepared and analysed by GC-MS and HPLC. An active ethyl acetate fraction obtained from methanol extract was tested on Gram-negative bacteria such as *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and a Gram-positive species *Bacillus subtilis*. An intestinal tapeworm, *Raillietina echinobothrida* was used for anthelmintic study and the effects were examined by scanning electron microscopy.

Results: GC-MS revealed that *N*-isobutyl-(2E, 4Z, 8Z, 10E)-dodecatetraenamide was the dominant compound in all the three extracts. *N*-(2-Methylbutyl)dodeca-2,4-diene-8,10-diynamide, *N*-(2-phenylethyl) non-2(E)-en-6, 8-diynamide and (2E,4E,10E)-*N*-isobutylhexadeca-2,4,10-trienamide were also detected. (2E,6Z,8E)-*N*-Isobutyl-2,6,8-decatrienamide was confirmed by HPLC in all extracts. The methanol-ethyl acetate extract was effective against all the four bacteria with maximum activity against *Bacillus subtilis*. Anthelmintic effects on *R. echinobothrida* included tegumental shrinkage, surface erosion, obliteration of the spines, and formation of pits on the body segments.

Interpretation: *A. oleracea* is shown to be rich in alkylamides. As the dominant compounds, these alkylamides can be attributed to the antibacterial and anthelmintic properties of the medicinal plant.

Keywords: *Acmella oleracea*, Alkylamide, Antibacterial, Cestode, Scanning Electron Microscopy



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Introduction

Acmella oleracea (R.K. Jansen) is a small perennial herb of the Asteraceae family which has received considerable attention because of its varied applications in different traditional practices and ethnomedicines (Paulraj *et al*., 2013). It is most popular for the remedy of toothache and it is formulated for commercial application in dental care. It is also patented as an anti-wrinkle cosmetic preparation in the form of creams and gels owing to its Botox-like muscle-smoothening effects, and for which it is aptly known as 'herbal Botox' (Tiwari *et al.,* 2011).

In different traditional medicines *A. oleracea* is used as an analgesic, antibiotic, anticonvulsant, antidiuretic, antifungal, anthelmintic, antiinflammatory, antiprotozoal, antipyretic, antiulcer, antiviral and also as an insecticide (Prachayasittikul *et al.,* 2013). It is a known remedy for impotency and also a strong aphrodisiac in Indian culture (Sharma *et al.,* 2011). It is further used for treating clinical conditions like cancer, snakebite, articular rheumatism and tuberculosis (Lalthanpuii *et al.,* 2018).

The aerial part is a common vegetable, consumed either raw or cooked among the tribal people of north-east India and Myanmar. Hence, it is widely cultivated and available throughout the year. In Mizo traditional medicine, the leaves are used for treating gastritis, while the flowers and leaves are for headaches, speech impediments, dental infection, rheumatism (Sawmliana, 2013), and it is uniquely applied in parasitic infections (Lalthanpuii *et al.,* 2017). It is therefore imperative that the chemical nature of the plant is understood with respect to the antiparasitic property. Thus, the aim of this study is to prepare different extracts of the plant, analyse the chemical composition by GC-MS and HPLC, and test the extract against bacteria and a parasitic tapeworm, *Raillietina echinobothrida.*

Materials and Methods

Plant extract: *Acmella oleracea* was harvested from Ngopa, Mizoram, India. The specimens (PUC-A-17-1) were identified at the Botanical Survey of India (BSI), Shillong, Meghalaya. The aerial parts consisting of the leaves and flowers were washed with distilled water, shade dried and were crushed to prepare a fine powder. Crude extracts were prepared in a 5-l Soxhlet apparatus using methanol, chloroform and hexane. The extracts were concentrated by evaporating and recovering the solvents in a vacuum rotary evaporator (Buchi Rotavapor® R-215), and then stored at 4°C until further use.

GC-MS: Volatile components of *A. oleracea* extracts were analysed in a single quadrupole gas chromatography-mass spectrometry system (Thermo Scientific TRACE™ 1300 ISQ™ LT). Acetonitrile was used as a solvent. A non-polar column TR-5MS (260F142P) of dimension 30 m x 0.25 mm x 0.25 µm and film thickness of 0.25 µm was used. The injector port, oven, ion source and transfer line temperature were set at 250°C. Helium was released @ 1 ml min^{-1}. One µl of the samples were injected in a splitting ratio of 1:50. The ionisation electron energy was set at 70 eV. The running duration was 55 min within the mass range 100-1000 m/z. The final chromatogram was generated with Thermo Scientific™ Xcalibur™ software. Compounds were identified from the libraries of Wiley Registry™ and National Institute of Standards and Technology database.

HPLC: Chemical fingerprinting was done using high performance liquid chromatography (Waters® 2489 UV/visible detector). A manual sample injector valve with 20 µm loop was used in which separation was achieved by reverse phase C18 column (5 µm, 4.6 x 250 mm). Standard isobutyl alkylamide (Santa Cruz Biotechnology, Dallas, Texas, U.S.A.) was used as a reference. Samples were dissolved in HPLC-grade solvents and filtered using Sep-Pak® C18 cartridge. Isocratic mode of elution was done at a flow rate of 0.5 $mlmin^{-1}$ using acetonitrile:water (93:7) as mobile phase. Each sample was run for 15 min and the eluted compounds were detected at 229 nm. The chromatograms were analysed using Empower2 software.

Antibacterial activity: Ethyl acetate fraction of methanol extract was highly active and was used for biological assays. The plant extract was first tested for antibacterial activity using Kirby-Bauer test. Four different bacteria were used such as *Pseudomonas aeruginosa* ATCC® 10145™ (Aerobic Gram-negative bacterium), *Klebsiella pneumoniae* ATCC® 10031™ (Anaerobic Gram-negative bacterium), *Escherichia coli* ATCC® 10536™ (Anaerobic Gram-negative bacterium), and *Bacillus subtilis* ATCC® 11774™ (Aerobic Gram-positive bacterium). The bacteria were grown in Mueller-Hinton agar. Plant extract at 10 and 20 $mgml^{-1}$ concentrations was impregnated on Whatman Antibiotic Assay Discs. Negative control contained only bacteria in agar medium, while positive control had 20 $mgml^{-1}$ of ceftriaxone. The assays were performed in triplicates. Bacteria were incubated at 37±1°C 20 hr. After incubation, the size of the inhibition zones was measured.

Antiparasitic activity: Tapeworms (*Raillietina echinobothrida* Mégnin, 1880) were recovered from the intestines of freshly sacrificed fowls, *Gallus gallus domesticus* Linnaeus, 1857. *A. oleracea* extract and albendazole (with a standard dosage of 20 $mgml^{-1}$) were prepared in different concentrations, *viz.* 5, 10 and 20 $mgml^{-1}$ in 0.9% neutral phosphate-buffered saline (PBS) with 1% DMSO. Control consisted only of PBS with DMSO. Batches of 2 worms were introduced into each media, and each test was carried out in triplicate. They were incubated at 37±1°C and their survival time was recorded. Data were normalised against those of the control and presented as mean ± SD. Data were subjected to Student's *t*-test and significance was considered at $p<0.05$.

Scanning electron microscopy: Tapeworms treated with 20 $mgml^{-1}$ of the plant extract were processed for scanning electron microscopy. They were fixed in 10% neutral-buffered

formaldehyde at 4°C for 4 hr. Dehydration was done in acetone. After treating with tetramethylsilane they were dried in an air-drying chamber at 25°C. After sputter coating with gold in JFC-1100 (JEOL Ltd., Tokyo, Japan) ion-sputtering chamber, they were observed under a JSM-6360 scanning electron microscope (JEOL Ltd., Tokyo, Japan) at an electron accelerating voltage of 20 kV.

Results and Discussion

GC-MS chromatogram of different extracts of *A. oleracea* are shown in Fig. 1-3. Analysis of the mass spectra revealed the presence of three alkylamides (Table 1). *N*-Isobutyl-(2E, 4Z, 8Z, 10E)-dodecatetraenamide was detected in all the extracts as the major compound, with a relative abundance of 92.66% in chloroform extract, 25.4% in methanol extract, and 67% in hexane extract. *N*-(2-methylbutyl)dodeca-2,4-diene-8,10-diynamide and (2E,4E,10E)-*N*-isobutylhexadeca-2,4,10-trienamide were detected only in the chloroform extract. *N*-(2-phenylethyl) non-2(E)-en-6, 8-diynamide was detected in the chloroform and methanol extract. HPLC fingerprinting using standard alkylamide indicated the presence of (2E,6Z,8E)-*N*-isobutyl-2,6,8-decatrienamide in all the extracts. The antibacterial activity of *A. oleracea* methanol-ethyl acetate extract was tested on four different bacteria, namely *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa* and *Bacillus subtilis* as shown in Table 2. The plant extract was effective against all the tested bacteria. The highest efficacy was noted on *Bacillus subtilis* in terms of inhibitory effect.

Table 3 shows the anthelmintic activities of albendazole and *A. oleracea* methanol-ethyl acetate extract on *R. echinobothrida*. Both the treatments showed concentration-dependent activity. At 5, 10 and 20 mgml^{-1}, albendazole took 23.76, 16.30 and 4.39 hrs to kill all the tapeworms; while plant extract took 37.36, 14.57 and 5.15 hrs, respectively.

Scanning electron microscopy revealed massive damages on the body of *R. echinobothrida* after treatment with 20 mgml^{-1} of *A. oleracea* methanol-ethyl acetate extract. Fig. 4 shows the anterior part of the tapeworm. The tegument was severely shrunk and eroded with large portions removed. Chunks of tissue remains adhered to the body at many places. A single attachment organ (sucker) showed severe deformity (Fig. 5). There were no sign of pointed spines, which are otherwise the

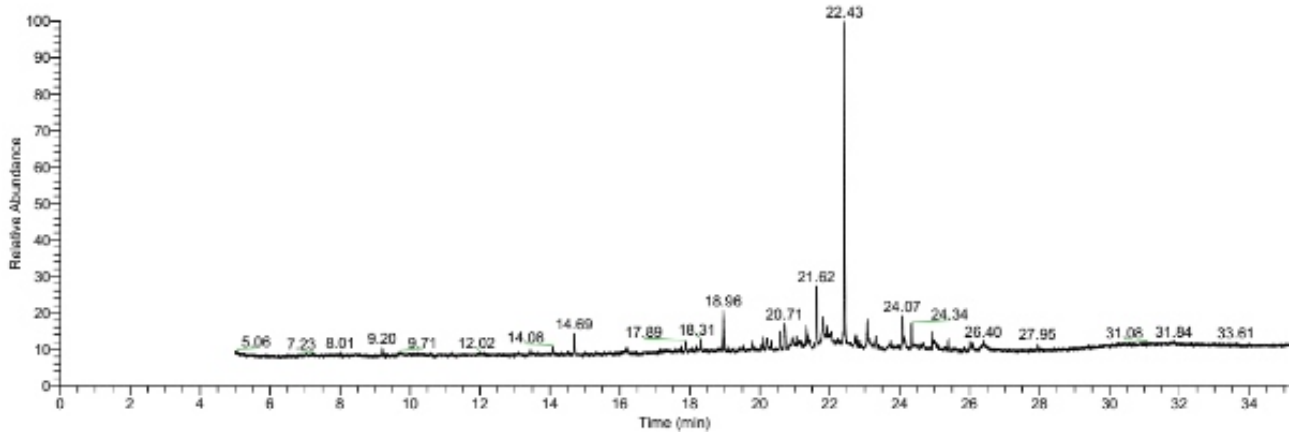

Fig. 1 : Gas chromatogram of methanol extract of *A. oleracea.*

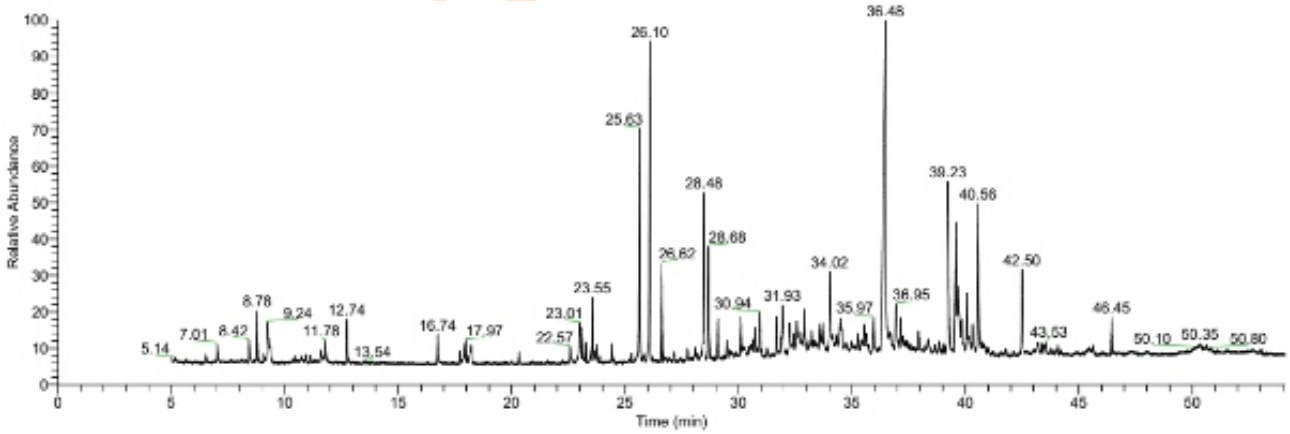

Fig. 2 : Gas chromatogram of chloroform extract of *A. oleracea.*

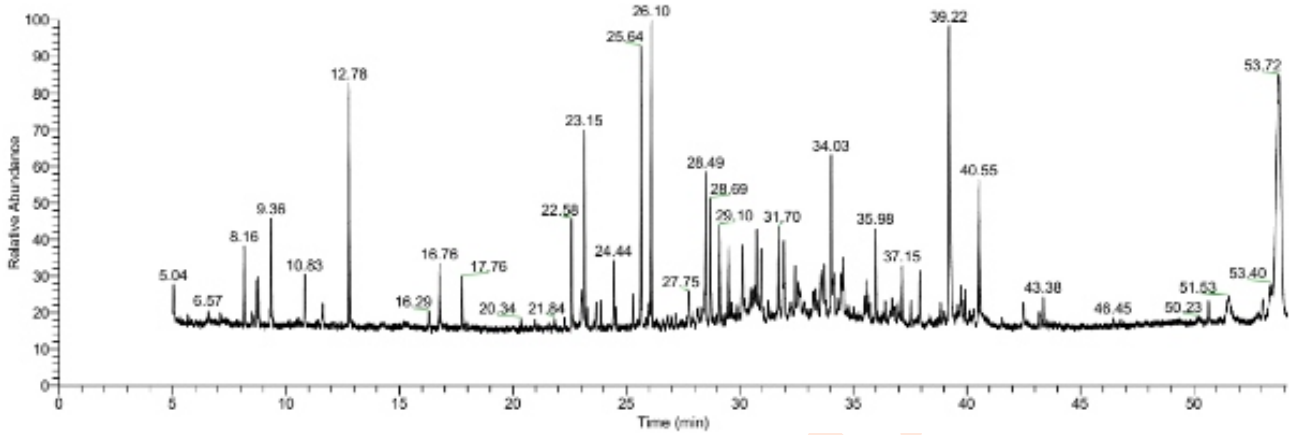

Fig. 3 : Gas chromatogram of hexane extract of *A. oleracea.*

Table 1 : Alkylamides detected from different extracts of *A. oleracea*

Compound	Molecular weight (Da)	Chemical formula	Methanolextract	Chloro-form extract	Hexane extract
N-Isobutyl-(2E, 4Z, 8Z, 10E)-dodecatetraenamide	247	$C_{16}H_{25}NO$	Present	Present	Present
N-(2-Methylbutyl)dodeca-2,4-diene-8,10-diynamide	257	$C_{17}H_{23}NO$		Present	
(2E,4E,10E)-*N*-Isobutylhexadeca-2,4,10-trienamide	305	$C_{20}H_{35}NO$		Present	
N-(2-Phenylethyl) no*N*-2(E)-e*N*-6, 8-diynamide	251	$C_{17}H_{17}NO$	Present	Present	
(2E,6Z,8E)-*N*-Isobutyl-2,6,8-decatrienamide	221	$C_{14}H_{23}$	Present	Present	Present

Table 2 : Antibacterial activity of ceftriaxone and *A. oleracea* methanol-ethyl acetate extract

Test material	Concentration[a]	Zone of inhibition (mm)[b] on			
		Escherichia coli	*Klebsiella pneumoniae*	*Pseudomonas aeruginosa*	*Bacillus subtilis*
Ceftriaxone	10 µg disk^{-1}	6.7	4.9	6.1	6.4
A. oleracea	0.5 mg disk^{-1}	2.1	2.1	2.0	2.5
	1.0 mg disk^{-1}	2.5	2.8	2.1	2.8

[a]Disk diameter was 6 mm; [b]average of three tests

Table 3 : Anthelmintic activity of albendazole and *A. oleracea* methanol-ethyl acetate extract on *R. echinobothrida*

Treatment	Concentration (mg ml^{-1})	Survival time (hr)[a]	*t*-value	*t*-critical value
Albendazole	5	23.76 ± 1.93*	58.32	2.26
	10	16.30 ± 0.66*	77.66	2.45
	20	04.39 ± 0.88*	86.57	2.45
A. oleracea extract	5	37.36 ± 2.73*	41.09	2.23
	10	14.57 ± 1.66*	68.64	2.26
	20	05.15 ± 1.40*	79.71	2.31

[a]Normalised values against those of control; * Significantly different at $p < 0.05$; $n = 6$.

main adhering devices to the host's intestinal lumen. The tegumental damage extended throughout the body, including body segments (proglottids) as shown in Fig. 6. Tissue debris were haphazardly attached, indicating progressive erosion of the tegument. Hairy microtriches were completely lost, instead minute pit-like holes were visible on all the proglottids. These pits indicate disintegration of microtriches and tegumental tissue. Alkylamides are a group of polyunsaturated fatty acids with substitute cyclic systems or heteromolecules such as nitrogen, sulfur and oxygen. As secondary metabolites present in about

Fig. 4 : Scanning electron microscopy of the anterior part of the body of *R. echinobothrida* treated with *A. oleracea* methanol-ethyl acetate extract (20 mg ml^{-1}). Extensive erosion of tegument and extreme contortion at the base of the neck.

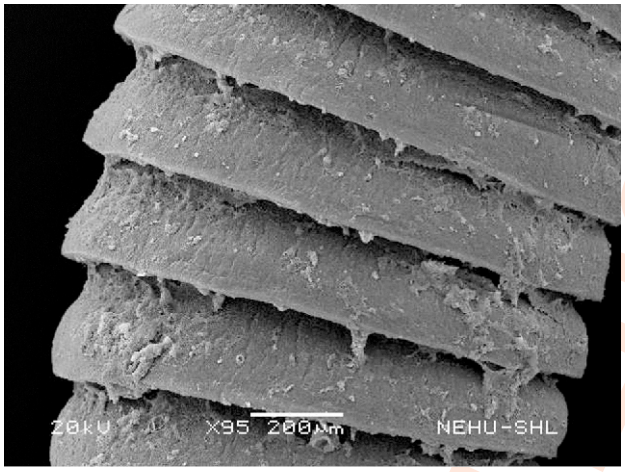

Fig. 6 : Scanning electron microscopy of the body segments of *R. echinobothrida* treated with *A. oleracea* methanol-ethyl acetate extract (20 $mgml^{-1}$). Tissue fragments are visible on all the segments, and small pit-like dots indicate degeneration of the tegument.

100 different medicinal plants, they are known to exhibit a wide range of biological activities including antibacterial, analgesic, antiinflammatory, antiprotozoal, insecticidal and immunomodulatory activities. They are attributed to various therapeutic uses of such as treatment for allergy, bronchitis, haemorrhoid, hyperglycaemia, hypertension, malaria, toothache, oral infection, gastric diseases, rheumatism, sexual dysfunctions and viral infections (Boonen *et al.*, 2012; Wynendaele *et al.*, 2018). Spilanthol is a unique alkylamide isolated from *A. oleracea*. It is accredited to most of the chemical and medicinal properties of the plant including pungency, tingling or burning sensation when the plant is eaten

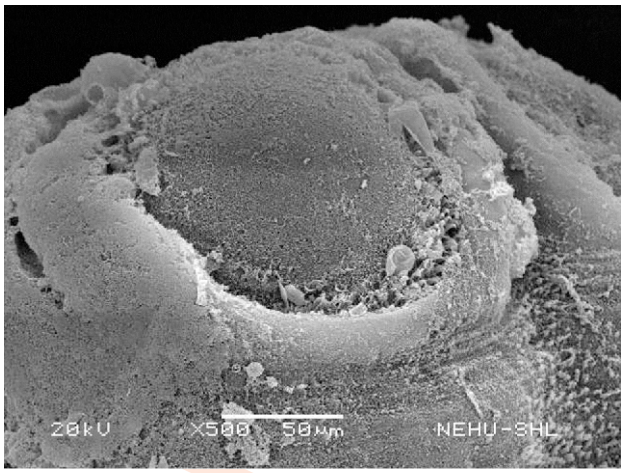

Fig. 5 : Scanning electron microscopy of the sucker of *R. echinobothrida* treated with *A. oleracea* methanol-ethyl acetate extract (20 $mgml^{-1}$). Sucker is bereft of pointy spines and its surrounding microtriches are reduced to fuzzy debris.

and Botox-like effects (Barbosa *et al.*, 2016; Silveira *et al.,* 2018). In view of the potentials of alkylamides as a source of novel antibiotics (Niu *et al.*, 2018; Chakthong *et al.*, 2019), this study adds to the compendium as *A. oleracea* extract was effective against a variety of pathogenic bacteria tested.

This suggests that alkylamides, as the major constituents, play a major role in the antibacterial activity. The body surface of helminth parasites are directly targeted by drugs as they are the immediate interface between the parasites and the host (Taman and Azab, 2014). In tapeworms, there are no digestive and nervous systems so that the tegument with its hair-like microtriches acts a functional primary absorptive and sensory organ. As such, the tegument is the most critical body part in the parasitic adaptation of tapeworms and for this reason it is always the primary target site of anthelmintic drugs (Rana and Misra-Bhattacharya, 2013). Albendazole and flubendazole reportedly caused rostellar damages, eruption of swellings or blebs on the tegument, defacement of the microtriches, and increased vesiculation on the tapeworm, *Echinococcus granulosus* (Elissondo *et al.,* 2006). It also caused severe contraction, removal of microtriches and tegumental collapse in *R. echinobothrida* (Lalchhandama, 2010). Markoski *et al.* (2006) reported that combined albendazole-praziquantel treatment of *Mesocestoides corti* resulted in extensive deformity of suckers, erosion of tegument and disintegration of microtriches.

It is, therefore, necessary to rationalise the anthelmintic efficacy of *A. oleracea* extract and its effect on the tapeworm substantiates of the traditional usage of this plant for helminthiasis. It is also important to identify the exact compound that exert anthelmintic activity.

Acknowledgment

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