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Short communication

Anthelmintic activity of praziquantel and *Spilanthes acmella* extract on an intestinal cestode parasite

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Spilanthes acmella Murr., popularised as toothache plant, is a well-known culinary and medicinal plant for different purposes, but its use as an anthelmintic is apparently exclusive to the Mizo people of India and Myanmar. A chloroform extract of *Spilanthes acmella* Murr. was analysed in a single quadrupole GC-MS system, from which it was found that the major compound was an alkylamide, *N*-isobutyl--(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide. A comparative study was performed on the anthelmintic activity of the plant extract and praziquantel (PZQ) against an intestinal cestode, *Raillietina echinobothrida*. In terms of efficacy, PZQ was more potent, but the plant extract was also effective at all concentrations tested. PZQ caused severe shrinkage and folds of the tegument, constriction of the suckers, dislocation of spines and erosion of microtriches. The plant extract caused shrinkage and folds on the main body but not on the scolex. Damage on the suckers is more pronounced than in PZQ--treated cestodes. The spines were completely removed. The current findings indicate that *S. acmella* is a good source of compounds with anthelmintic activity.

Keywords: *Spilanthes acmella*, anthelmintic, GC-MS, *Raillietina echinobothrida*, scanning electron microscopy

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The medicinal plant *Spilanthes acmella* Murr. (family *Asteraceae*) is known for a variety of its medicinal and culinary usages in different cultures (1). It is most famous for its use in dental health care for which it earned a common name as toothache plant (2). It is often cooked as a vegetable or used raw as food seasoning because of its characteristic menthol-like minty flavor. It is among the most versatile medicinal plants such as in the treatment of blood disorder, cancer, constipation, diuresis, pyrexia, flatulence, inflammation, hepatic abscess, peptic ulcer, and other ulcerations (3, 4). In Indian and some African cultures, it is also regarded as a good therapy for severe malaria (5). In Indian medicine, it is claimed as an effective remedy for impotency and as an aphrodisiac (6). In addition, it is also used for treating articular rheumatism, dysentery, snakebite and tuberculosis (7).

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S. acmella has been experimentally validated for analgesic, antimicrobial, antioxidant, diuretic, larvicidal and mosquitocidal activities (3). It specifically induced increased proliferation of macrophages in the blood, which indicates its ability to enhance the immunomodulatory activity of phagocytes (8). It can also suppress inflammation due to the activation of neutrophils in the lungs in experimental mice (9). Its efficacy in reducing the level of inflammation in Brewer's yeast-induced pyrexia further substantiates its use in the treatments of high fever and inflammation (10). It also exhibits insecticidal activities against the insect-pest *Tuta absoluta* (11), and vectors of important infectious diseases including *Aedes aegyptii* (12), *Anopheles* and *Culex* species (13). The pungent odour is useful to ward off mosquitos and other insects. In addition, the juicy extract is used for poisoning (14). Perhaps, the most exclusive application among the Mizo people is as a deworming agent for intestinal helminthiases and is asserted as effective against both cestode and nematode parasites. This interesting medicinal usage is the basis of this study, to assess whether the plant extract possesses the broad-spectrum anthelmintic property.

EXPERIMENTAL

Chemicals and drugs

All chemicals of standard analytical grade were procured from HiMedia Laboratories, India. Acetonitrile was a product of Merck Life Science, India. Praziquantel was a product of Astra Lifecare, India.

Preparation of plant extract

S. acmella was harvested from Ngopa, a village in Champhai district, Mizoram, India, located between 23 8861°N and 93 2119°E. The specimen was identified and authenticated at the Botanical Survey of India, Shillong, India, and is catalogued (with accession no PUC-A-17-1) in the herbarium section of Pachhunga University College, Aizawl, Mizoram, India. The aerial parts of the plant were washed with distilled water and dried in shade. Extraction was done in a 5-litre Soxhlet apparatus using chloroform as the solvent. The extraction was complete after 72 hours. The slurry of the extract was concentrated in a vacuum rotary evaporator under reduced pressure. The semi-solid extract was obtained and was stored in a refrigerator at 4 °C until use.

Chemical analysis

The components of *S. acmella* chloroform extract were analyzed in a gas chromatography--mass spectrometry system (Thermo Scientific TRACE™ 1300 ISQ™ LT, USA). The plant extract was dissolved in acetonitrile. The stationary phase consisted of non-polar column TR-5MS (260F142P) having a dimension of 30 m × 0.25 mm with a film thickness of 0.25 μm. The injector port was set at 250 °C, whereas the oven was initially set at 70 °C for 2 minutes and incrementally increased by 10 °C up to 250 °C. Helium was released at a constant flow rate of 1 mL min^{-1} into the oven chamber. One μL of the sample was injected in a split mode at the splitting ratio of 1:50. The ionisation electron energy of the mass spectrometer was maintained at 70 eV. The temperatures of the ion source and transfer line were set at 250 °C.

The total running duration was 55 minutes. The final chromatogram and mass spectra were generated with Thermo Scientific™ Xcalibur™ software. Compounds were identified on the basis of their retention times, chemical formula and molecular mass from the libraries of Wiley Registry™ 10 and National Institute of Standards and Technology database.

Anthelmintic test

Anthelmintic activity was tested *in vitro* on a cestode (tapeworm) *Raillietina echinobothrida* Mégnin, 1880. Live parasites were obtained from the intestines of naturally infected and freshly sacrificed local fowls, *Gallus gallus domesticus* L., 1758. The study was approved by the Ethical Committee of Pachhunga University College (PUC-IAEC-2016-Z2 of 10/08/2016).

Plant extract and praziquantel solutions were prepared in 0.9 % neutral phosphate-buffered saline (PBS) supplemented with 1 % dimethylsulfoxide (DMSO) in concentrations of 1.25, 2.5, 5, 10 and 20 mg mL^{-1}. The control consisted of PBS with 1 % DMSO. A set of two worms was introduced into each medium and each test was done in triplicate. They were incubated at 37 ± 1 °C.

Anthelmintic efficacy was assessed in terms of duration of survival in the culture media. Death was confirmed when worms failed to show any sign of movement after stimulation by dipping in tepid PBS (45 °C). The time of death was recorded.

Statistical analysis

Treatment data were normalized to the survival time of cestodes in the control experiment, *i.e.*, 72.03 hours. They were expressed as mean ± standard deviation. Student's *t*-test was used to analyse the data and the level of significance was considered when the *p*-value was less than 0.05.

Scanning electron microscopy

For scanning electron microscopy, cestodes treated with 20 mg mL^{-1} of praziquantel and the plant extract were used. The worms were fixed in 10 % cold-buffered formaldehyde at 4 °C for 4 hours. The fixative was buffered with 0.1 mol L^{-1} sodium cacodylate (pH 7.2). Osmium tetroxide (OsO_4), 1 %, was used as a secondary fixative. The specimens were then dehydrated through grades of acetone up to pure acetone. They were then immersed in tetramethylsilane, $Si(CH_3)_4$, for 15 minutes and left to dry in an air-drying chamber at 25 °C. They were mounted on metal stubs and sputter-coated with gold in JFC-1100 (Jeol, Japan) ion-sputtering chamber. Finally, they were observed under a JSM-6360 scanning electron microscope (Jeol) at an electron accelerating voltage of 20 kV.

RESULTS AND DISCUSSION

Chemical analysis

The gas chromatogram of *S. acmella* chloroform extract is shown in Fig. 1, and the corresponding list of compounds identified from it are presented in Table I. Twenty-two major peaks representing 20 different compounds were identified. The most abundant was an

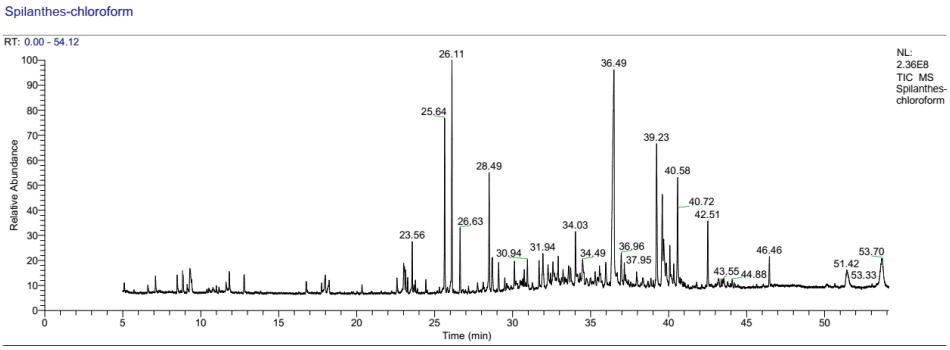

Fig. 1. Gas chromatogram of the chloroform extract of *S. acmella.* The total retention time is 55 minutes.

alkylamide, *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide, which was detected at two peaks with the relative abundance of 69.22 and 92.66 %. Another alkylamide, *N*-(2-phenylethyl) non-2(*E*)-en-6,8-diynamide, was also present in a fair amount (48.04 %). Other major compounds were: oplopanone or 1-((1*S*,3a*R*,4*R*,7*S*,7a*S*)-4-hydroxy-7-isopropyl-4-methyloctahydro-1*H*-inden-1-yl) ethanone, 2-hexadecen-1-ol-3,7,11,15-tetramethyl-phytol, pentanoic acid and *n*-hexadecanoic acid.

Chemically, *S. acmella* is known to contain several important compounds including α- and β-amyrinester, amides, miricilic alcohol glycosides, saponins, sitosterol, stigmasterol and triterpenes (15). These compounds have attributed a variety of pharmacological properties. In fact, alkylamides are established to be the principal bioactive compounds in the genus *Spilanthes.* Specifically, *N*-isobutyl-2*E*,6*Z*,8*E*-decatrienamide is identified to be the main alkylamide in the genus *Acmella*, *Heliopsis longipes* and *Welelia parviceps,* and is attributed to most of the biological activities of these plants, including their pungent insect-repellent and minty properties. The compound has been experimentally shown to have analgesic, anti-cancer, anti-inflammatory, antimicrobial, antioxidant, antimutagenic, neuroprotective and insecticidal activities (16).

It is interesting that an alkylamide, *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide, was found to be the major compound in *S. acmella*. This and the other alkylamide, *N*-(2--phenylethyl) non-2(*E*)-en-6,8-diynamide, are also reported in other *Spilanthes* species (17). Hexadecanoic acid (palmitic acid) detected in this study is already known to exhibit anti-inflammatory activity (18). Phytol reportedly showed antimicrobial, cytotoxic, antimutagenic, antidiabetic, antispasmodic, anticonvulsant, antinociceptive, anti-inflammatory, antidepressant and hair-growth facilitating activities (19). It is therefore conceivable that some of these compounds are responsible for the anthelmintic activity of *S. acmella.*

Anthelmintic activity

The anthelmintic efficacy of praziquantel and the chloroform extract of *S. acmella* on the cestode, *R. echinobothrida*, is given in Table II. Significant concentration-dependent effects were seen for all the tests. Praziquantel was more effective than the chloroform extract of *S. acmella*.

Table I. Compounds identified in S. acmella chloroform extract[a]

Compd. No.	t_R (min)	Relative abundance (%)	Compound	Formula	M_r
1	23.56	12.35	9-Hexadecen-1-ol	$C_{16}H_{32}O$	240
2	25.64	4.90	(*Z*)6-Pentadecen-1-ol	$C_{15}H_{30}O$	226
3	26.11	6.85	9-Hexadecen-1-ol	$C_{16}H_{32}O$	240
4	26.63	65	2,4-bis(1,1-Dimethylethyl)phenol	$C_{14}H_{22}O$	206
5	28.49	35.90	Caryophyllene oxide	$C_{15}H_{24}O$	220
6	30.94	13.01	Perhydrocyclopropa[*e*]azulene-4,5,6-triol, 1,1,4,6-tetramethyl	$C_{15}H_{26}O_3$	254
7	31.94	64.88	1-((1*S*,3a*R*,4*R*,7*S*,7a*S*)-4-hydroxy-7-isopropyl-4-methyloctahydro-1H-inden-1-yl) ethanone	$C_{15}H_{26}O_2$	238
8	34.03	52.61	6,10,14-Trimethyl-2-pentadecanone	$C_{18}H_{36}O$	268
9	34.49	9.02	1-Heptatriacotanol	$C_{37}H_{76}O$	536
10	36.49	67.22	*n*-Hexadecanoic acid	$C_{16}H_{32}O_2$	256
11	36.96	38.34	Ethyl hexadecanoat	$C_{18}H_{36}O_2$	284
12	37.95	50.92	*N*-[4-[[(2-amino-1,4-dihydro-4-oxo-6-pteridinyl)methyl]amino]benzoyl]	$C_{19}H_{19}N_7O_6$	441
13	39.23	69.29	2-Hexadecen-1-ol-3,7,11,15-tetramethyl-phytol	$C_{20}H_{40}O$	296
14	40.58	92.66	*N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide	$C_{16}H_{25}NO$	247
15	40.72	52.96	*N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide	$C_{16}H_{25}NO$	247
16	42.51	48.04	*N*-(2-phenylethyl) non-2(*E*)-en-6,8-diynamide	$C_{17}H_{17}NO$	251
17	43.55	16.03	Pregn-4-ene-3,30-dione-17,21-dihydroxy-bis(*o*-methyloxime)	$C_{23}H_{36}N_2O_4$	404
18	44.08	76.10	2,4,6,8,10-Tetradecapentanoic acid	$C_{36}H_{46}O_8$	606
19	46.46	19.42	Diisooctyl phthalate	$C_{24}H_{38}O_4$	390
20	51.42	33.31	Cholesta-22,24-dien-5-ol-4,4-dimethyl	$C_{28}H_{48}O$	412
21	53.33	54.83	9-Desoxo-9-x-acetoxy-3,8,12-tri-*o*-acetyl-ingol	$C_{28}H_{40}O_{10}$	536
22	53.70	43.57	4,4,6a,6b,8a,11,11,14b-Octamethyl-1,4,4a,5,6,6a,6b,7,8,8a,9,10,11,12,12a,14,14a,14b-octadecahydro-2*H*-picen-3-one	$C_{30}H_{48}O$	424

[a] Retention time was 55 minutes; *m*/*z* range was 100–1000 Da.

Table II. In vitro *survival test of the cestode,* Raillietina echinobothrida, *upon treatments with praziquantel and* S. acmella *chloroform extract*

Treatment	Concentration (mmol L^{-1})	Normalised survival value (h)[a]	*t*-value	*t*-critical value
Control (PBS)	0	100.00 ± 2.56	NA	NA
Praziquantel	0.004	9.51 ± 0.37*	81.63	2.45
	0.008	6.79 ± 1.05*	82.56	2.36
	0.016	2.83 ± 0.74*	89.38	2.45
	0.032	1.29 ± 0.33*	93.76	2.57
	0.064	0.73 ± 0.10*	95.01	2.57
S. acmella[b]	0.005	72.13 ± 0.94*	25.07	2.45
	0.010	62.09 ± 1.35*	32.10	2.31
	0.020	59.40 ± 1.67*	32.56	2.26
	0.040	43.99 ± 1.52*	46.15	2.31
	0.081	31.35 ± 2.05*	51.10	2.23

[a] Mean ± SD, $n = 6$ (two worms in triplicate).
[b] Concentration calculated with respect to the most abundant compound, *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide.
* Significantly different at $p < 0.05$ in comparison with control.
NA – not applicable

Scanning electron microscopic images of *R. echinobothrida* after treatment with praziquantel are shown in Figs. 2–4. In Fig. 2, the anterior portion of the body is seen with severe shrinkage and tegumental folds in the scolex and neck region. Suckers are constricted to irregular shapes. A single sucker indicates massive erosion of the tegument while the spines are dislodged (Fig. 3). The general body (strobila) consisting of chains of unusual puffy segments (proglottids) is shown in Fig. 4. Tegumental folds are also visible. The shrinkage and folding are all over the segments, indicating a complete loss of hairy-absorptive filaments (microtriches) and extensive disintegration of the tegument.

S. acmella extract also caused considerable tegumental damages which are noticeably different from those caused by praziquantel. The scolex is severely deformed but without folds. Instead, the tegument appears to be hardened and eroded (Fig. 5). In Fig. 6, complete destruction of a sucker is visible. Microtriches and spines are completely obliterated and leave an empty pit. Tegumental shrinkage is seen on the proglottids (Fig. 7). Shrinkage is accompanied by severe folds and complete erosion of microtriches on the mature proglottids.

The molecular mechanism of action of praziquantel, one of the most commonly used anthelmintics, is still a mystery. Its primary target of helminth tegument posits that it may interfere with the proteins of the parasite body coverings (20). We have noted severe tegumental damages on the cestode such as sloughing of the suckers, shrinkage and erosion in praziquantel-treated *R. echinobothrida.* These effects are in agreement with other studies that

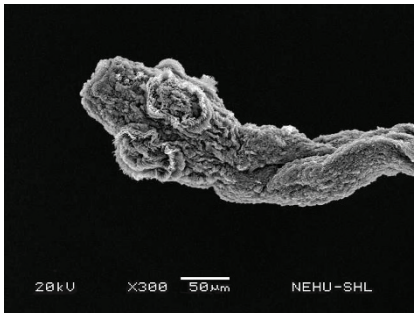

Fig. 2. Scanning electron microscopic image of the anterior portion of *R. echinobothrida* treated with praziquantel. The scolex with two suckers and an apical rostellum and the neck are shrunk and wrinkled. Magnification 300×, scale bar = 50 μm.

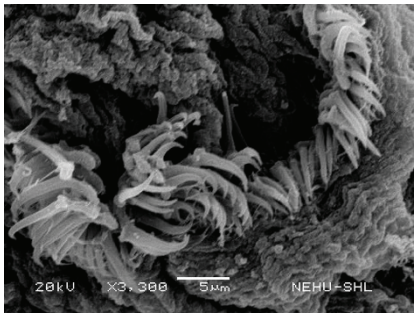

Fig. 3. Magnified view of a sucker of *R. echinobothrida* treated with praziquantel. The surrounding tegument is damaged and the spines are detached. Magnification 3300×, scale bar = 5 μm.

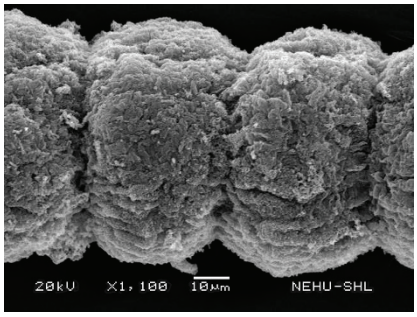

Fig. 4. The main body (strobila) of *R. echinobothrida* treated with praziquantel. The body segments (proglottids) are swollen and microtriches are lost. Magnification 1100×, scale bar = 10 μm.

showed similar damaging effects. Disintegration, sloughing, and erosion of the tegument have been described in *Schistosoma mansoni* after treatment with praziquantel (21). Praziquantel-resveratrol combination caused damages in the tegumental and subtegumental tissues

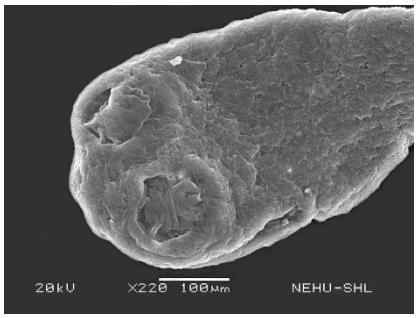

Fig. 5. Scanning electron microscopic image of the anterior portion of *R. echinobothrida* treated with *S. acmella* extract. Complete damage of the tegument. Suckers are visible as hollow and empty pits. Magnification 220×, scale bar = 100 μm.

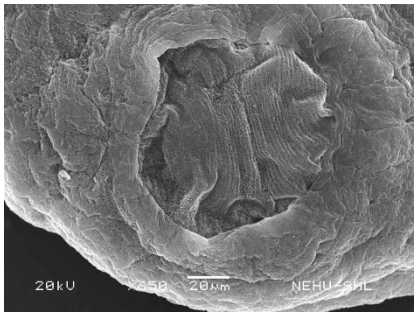

Fig. 6. A single sucker of *R. echinobothrida* treated with *S. acmella* extract. Complete loss of microtriches and spines. Magnification 650×, scale bar = 20 μm.

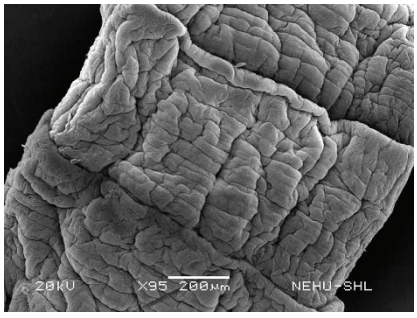

Fig. 7. Strobila of *R. echinobothrida* treated with *S. acmella* extract. Proglottids are shrunk and folded, and microtriches are completely removed. Magnification 95×, scale bar = 2.

of *S. mansoni* (22). Shrinkage of the tegument, rostellar edema and complete loss of hooks were observed in *Hymenolepis nana* (23). Piplartine, an amide from *Piper tuberculatum*, also caused tegumental destruction in the oral and ventral sucker regions of *S. mansoni* (24).

CONCLUSIONS

We demonstrated that a variety of *S. acmella* cultivated by the Mizo people exhibits anthelmintic activity with specific efficacy and damaging effects. The detrimental effects on the body of *R. echinobothrida* affirm the promising anthelmintic property as used in traditional medicine. This further implies that the precise nature of the anthelmintic effect is different from known anthelmintics, and thus, includes a different mechanism of action. As the plant extract is rich in *N*-alkylamides, particularly *N*-isobutyl-(2*E*,4*Z*,8*Z*,10*E*)-dodecatetraenamide, it is tempting to suggest that the anthelmintic activity is largely, if not entirely, due to this compound. Hence, this study poses a compelling reason for further investigations of *S. acmella* regarding the isolation of the anthelmintic compound and its mechanism of action.

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