SUPPLEMENTARY MATERIAL

Ultrastructural changes in *Raillietina* (Platyhelminthes: Cestoda), exposed to Sulfonoquinovosyldiacylglyceride (SQDG), isolated from Neem (*Azadirachta indica*)

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Abstract

Neem (*Azadirachta indica*), has been known to be a curative for various ailments and diseases in the traditional Indian medicinal system from times immemorial. A glycolipid sulfonoquinovosyldiacylglyceride (SQDG) isolated from the leaves of neem has been found to be a proactive antibacterial and antiviral agent in previous studies. The current communication pertains to the anthelmintic activity of SQDG *in vitro* against a model cestode *Raillietina* spp. The results of efficacy tests showed a paralysis time of 1.0 ± 0.1 and 0.7 ± 0.01 hour whereas death time of 1.6 ± 0.3 and 0.9 ± 0.02 hour, following treatments with dosages of 0.5 mg/ml and 1.0 mg/ml respectively. The scanning electron microscopic studies showed significant and unique changes in the ultrastructure of the worms with prominent breakages and furrows on the surface.

Keywords – Anthelminthic, *Azadirachta indica*, Sulfonoquinovosyldiacylglyceride, *Raillietina*, anticestodal efficacy, SEM

Experimental

Methods for isolation of Raillietina spp.

The mature *Raillietina* spp. were collected on autopsy from the intestine of freshly slaughtered fowls in 0.9% phosphate buffered saline (PBS: NaCl 8g; KH_2PO_4 0.34g and K_2HPO_4 1.21g in one litre of distilled water, pH: 7-7.3) from the local abattoirs.

Plant material

Mature leaves of *A. indica* were collected from the medicinal plant garden of R. K. Mission, Narendrapur, Kolkata, during January 2013 and identified by Dr. Ambarish Mukherjee, Professor of Botany, Burdwan University West Bengal. A voucher specimen (No.112) was deposited in the Chemistry department, Indian Institute of Chemical Biology, Kolkata.

General procedures

^{1}H and ^{13}C NMR spectra were recorded at 600 and 150 respectively using a Bruker DPX 600 MHz spectrometer in DMSO with TMS as internal standard. ESI-TOF mass was performed on a Q-TOF-Micromass spectrometer. IR spectra were recorded as KBr pellets using a JASCO 7300FTIR spectrometer. Optical rotations were measured on a JASCO DIP-370 digital polarimeter. Silica gel (silica gel 60-120, Merck) and Silica gel (100-200, Merck) were used for column chromatography. TLC was carried out on silica gel 60 F_{254}, and spots were visualized by spraying with Lieberman–Burchard is a reagent followed by heating.

Extraction, Isolation and characterization

As mentioned in Chatterjee et al and Bharitkar et al. 2010, 2014, the leaves of *A. indica* were cut into small pieces and air dried at room temperature. The dried leaves (2 kg) were defatted with petroleum ether (60-80°C) for 24 h and extracted thrice with methanol (5x3 lit) for 48 h each time at ambient temperature. The methanol extract was filtered and the solvent was dried under vacuum at 40-45°C to afford 300 g of crude extract (yield 15%) (Bharitkar et al., 2013, 2014). The compound SQDG was isolated as described (see Fig. 1) and characterized by the application of various spectroscopic analyses like IR, ESI-MS, ^{1}H NMR, ^{13}C NMR with DEPT 90 and 135, 2D NMR– COSY, NOESY, HSQC and HMBC and by comparison of its spectroscopic data with those reported in Sassaki et al., 2001. Details of chemical shift values of assignment ^{1}H NMR and ^{13}C NMR were given in the **table-S1**.

SQDG:$[\alpha]_D$ +39.2 (*c* 0.5, MeOH);IR (KBr, $\nu_{max}cm^{-1}$) 3458, 2919, 2851, 1736, 1467, 1374, 1174 and 1060; ^{1}HNMR (DMSO-d_6, 600 MHz) 0.84 (t, *J*=6.6 Hz), 1.22 (m), 1.48 (m), 2.25 (m), 2.31 (m), 2.55 (dd, *J* =6.6, 13.8 Hz), 2.90 (m), 3.18 (m), 3.77 (m), 3.88 (dd, *J* = 6.0, 10.2 Hz), 4.13 (dd, *J* = 7.8, 11.4), 4.34 (d, *J* =10.8 Hz), 4.57 (d, *J* =3.0 Hz), 4.67 (d, *J* =6.0Hz), 4.78 (d, *J* = 4.2 Hz), 5.13 (m), 5.39 (m); ^{13}CNMR (DMSO-d_6, 150 MHz) 13.9, 22.2, 24.5, 27.7, 28.2, 28.6, 28.8, 28.9, 29.0, 29.1, 29.2, 31.4, 33.5, 33.6, 40.0, 54.5, 62.7, 64.6, 68.6,

69.7, 71.6, 72.9, 74.2, 98.3, 172.4, 172.5. MS [ESI-MS, positive mode]: 839.51 $[M+Na]^+$, 817.53 $[M+H]^+$.

Treatments

The mature worms were incubated at 37±1°C in media containing varying dosages of the plant materials in the ascending order, in PBS with 0.1% DMSO. The broad-spectrum anthelmintic Praziquantel (Hoechst India Ltd.) was used as the reference drug in appropriate doses with respect to the plant materials. A set of petri dishes with 0.1% DMSO in PBS was maintained as control for each concentration. A particular concentration was tested with three replicates, each containing a batch of three worms with approximately the same size, weight and maturity. The time required for the onset of paralysis and death of the parasites was noted. The permanent immobilization of treated and control worms were determined visually when no motility occurred on physically disturbing them. The confirmation of death was done by dipping the parasite in slightly warm water at 40-50°C, which induced movements in the worm, still alive.

The paralyzed cestode (after treatment with SQDG) was processed for further studies, only the selected dosages of the SQDG were chosen for the purpose of electron microscopic study. At these doses the onset of the paralytic state in the parasite could be attained in a relatively short span of time that compared well with the timings of the reference drug, praziquantel.

Anthelmintic efficacy was determined in terms of motility, survivability, and histomorphological changes if any, in the treated worms.

Scanning Electron Microscopy (SEM)

For SEM preparation the samples are fixed in 10% neutral buffered formalin and then preparation of samples for SEM observation was followed as described by Ash et al. (2012); briefly the specimens were dehydrated through a graded ethanol series, transferred to hexamethyldisilazane (HMDS), dried in air, sputtered with gold (approximately 10 nm thick) and examined with a Jeol JSEM 7401F microscope.

Table S1: ^{1}H and ^{13}C chemical shift value (in CD_3OD) of compound

Carbon no.	**δ (H)**	**δ (C)**
C-1a, Gly	3.90 (m)	64.6
C-1b	3.29 (m)	
C-2, Gly	5.13 (m)	69.7
C-3a, Gly	4.34(d, *J*=10.8 Hz)	62.6
C-3b	4.13(dd, *J*=10.8, and 11.4 Hz)	
C-1'	4.68(d, *J*=3 Hz)	98.3
C-2'	3.29 (m)	72.9
C-3'	2.91 (m)	74.2
C-4'	3.18 (m)	71.6
C-5'	3.77 (m)	69.7
C-6'	2.91 (m), 2.55 (m)	54.5
1'', 1''' (C=O)	-	173.5, 173.4
2''-15'', 2'''-15''' (CH_2)	1.15-2.32 (m)	22.1-33.6
16'', 16''' (CH_3)	0.84 (t, *J*=6.6 Hz)	13.9

Table-S2: Anthelmintic efficacy results

Sample	Doses	Time (h) taken for*	
		Paralysis	**Death**
SQDG	0.5mg/ml	1.0±0.1	1.6±0.3
	1 mg/ml	0.7±0.01	0.9±0.02
Methanol ext.	10 mg/ml	1.5±0.02	3.2±0.02
	15mg/ml	1.3±0.02	2.0±0.02
Praziquantel	0.01mg/ml	0.5±0.01	7.2±0.1
	0.001mg/ml	3.0±0.01	8.9±0.2

*Data represent mean values six standard mean deviation (SEM) of three experiments. Student's t-test insignificant. Worms incubated in control medium showed physical activity till 72 ± 0.05 h.

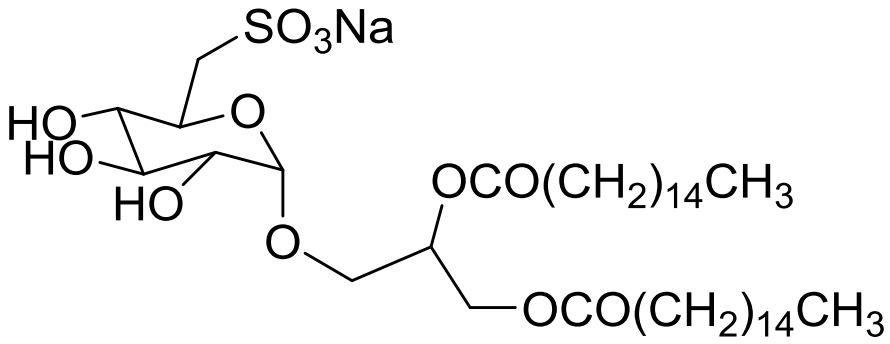

Figure S1. Structure of Sulfonoquinovosyldiacylglyceride (SQDG).

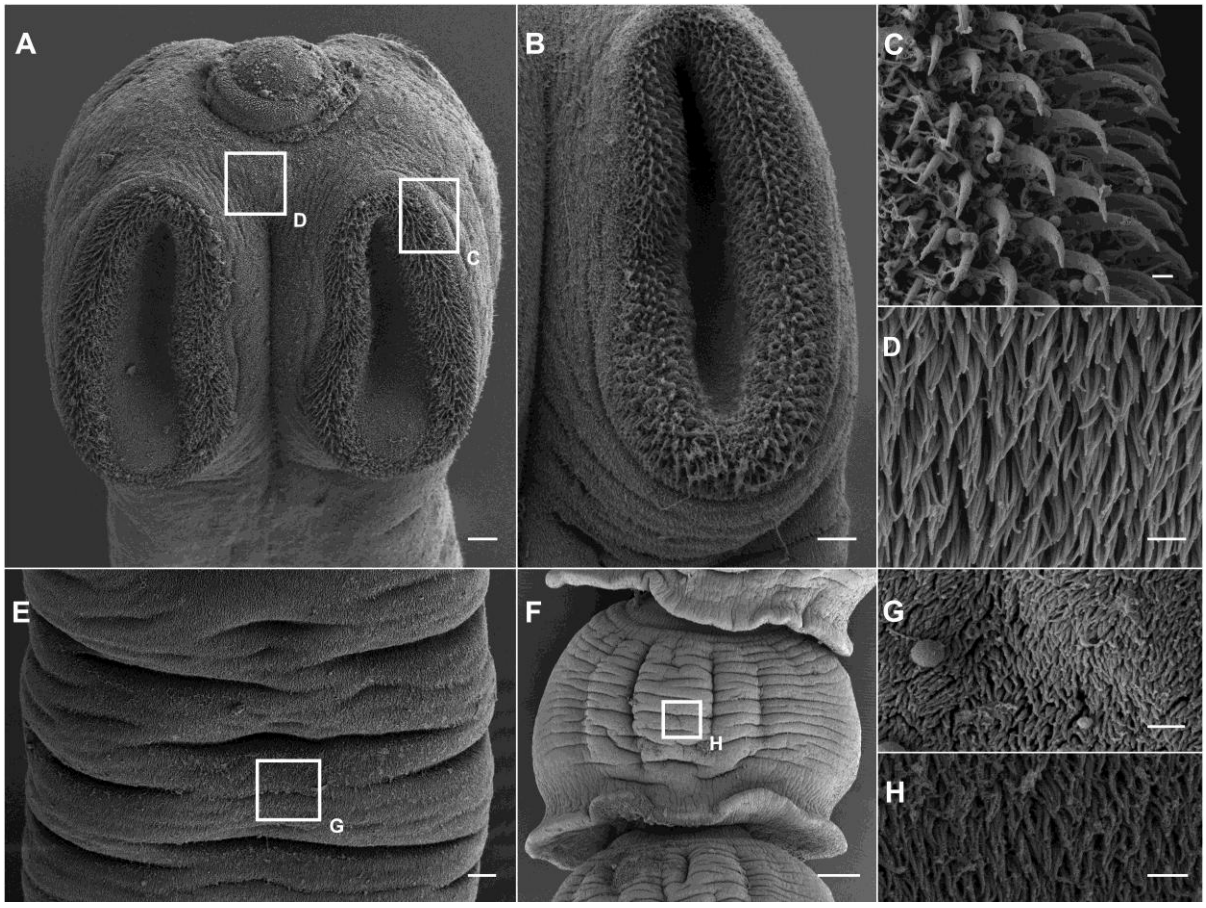

Figure S2. Scanning electron micrographs of control *Raillietina* spp.: (A) Scolex showing retractable rostellum and four suckers (scale bar =10 µm). (B) Sucker rim showing circlets of hooklets (scale bar =10 µm). (C) Details of hooklets and microtriches on sucker (scale bar =1 µm). (D) Enlarged view of a portion in between suckers showing small gladiate spinitriches (scale bar =1 µm). (E) Part of neck region (scale bar =10 µm). (F) Mature proglottid (scale bar =100 µm). (G) Enlarged view of a portion of neck region showing acicular filitriches

(scale bar =1 μm). (H) Enlarged view of a portion of proglottid showing small gladiate spinitriches (scale bar =1 μm).

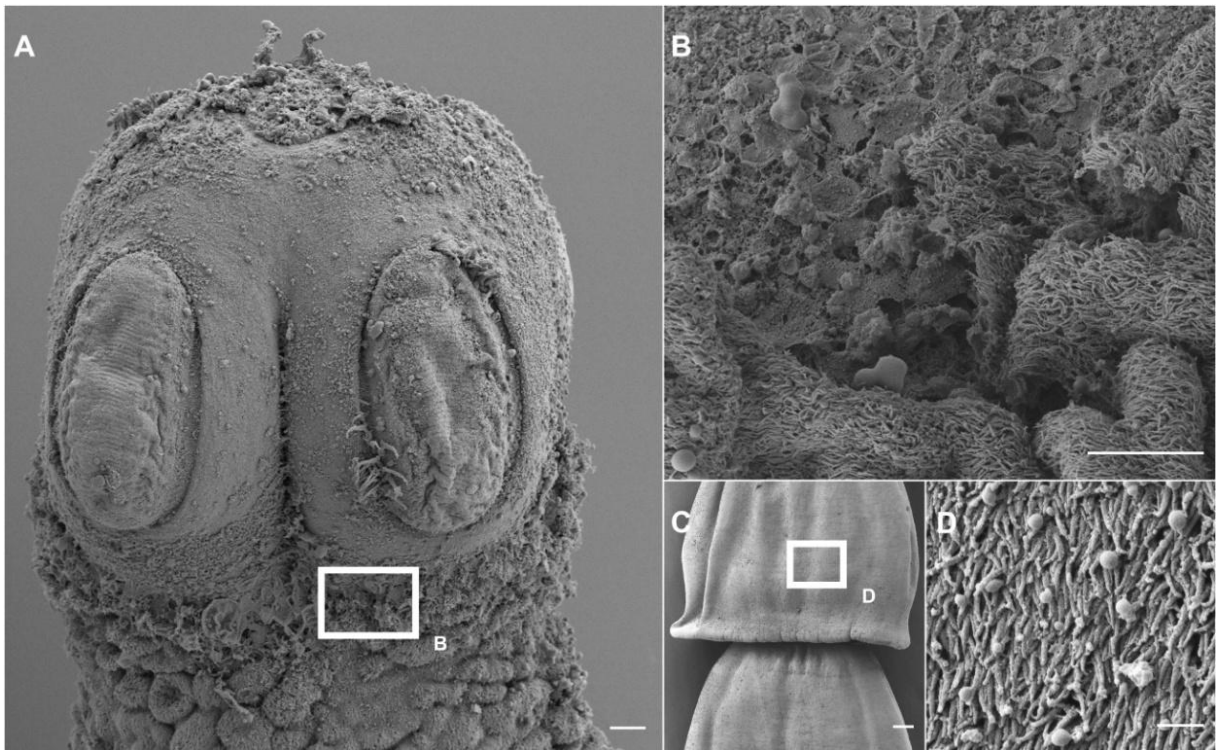

Figure S3. Scanning electron micrographs of *Raillietina* spp. treated with methanol extract of *A. indica* (Neem) (10 mg/ml): (A) Scolex and neck region; note damaged suckers where hooklets were mostly broken and fallen off, and with shredded microtriches (scale bar =10 μm). (B) Enlarged view of neck region; note shredded microtriches (scale bar =10 μm). (C) Mature proglottid (scale bar =100 μm). (D) Enlarged view of a portion of proglottid; note unchanged small gladiate spinitriches (scale bar =1 μm).

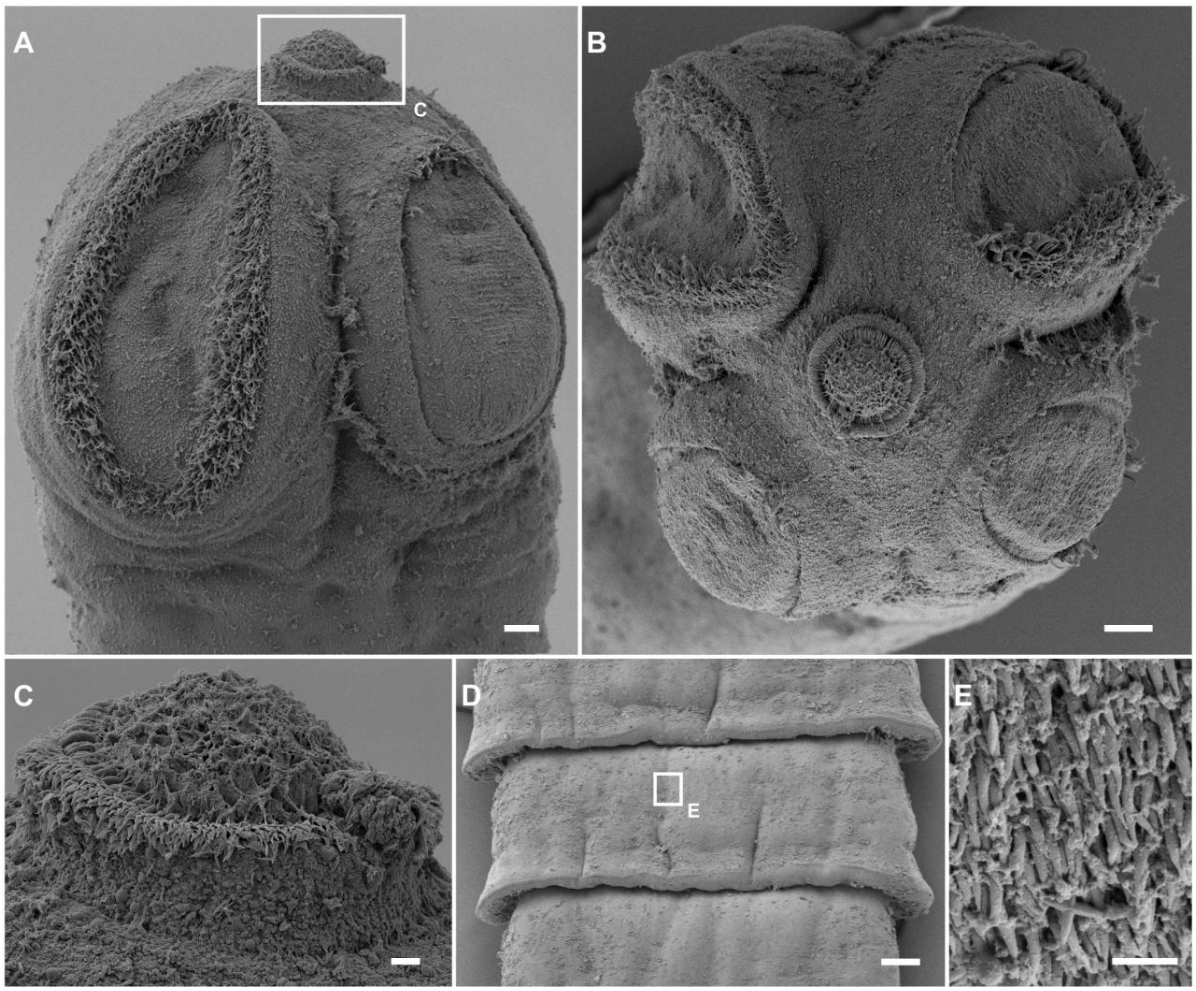

Figure S4. Scanning electron micrographs of *Raillietina* spp. treated with SQDG (0.5 mg/ml): (A) Scolex and neck region; note one damaged suckers where hooklets were mostly broken and fallen off, and shredded microtriches (10 μm). (B) Apical view of scolex; note shredded microtriches and damaged suckers (10 μm). (C) Enlarged view of rostellum; note shredded microtriches (scale bar =2 μm). (D) Mature proglottid (scale bar =100 μm). (D) Enlarged view of a portion of proglottid; note unchanged small gladiate spinitriches (scale bar =1 μm).

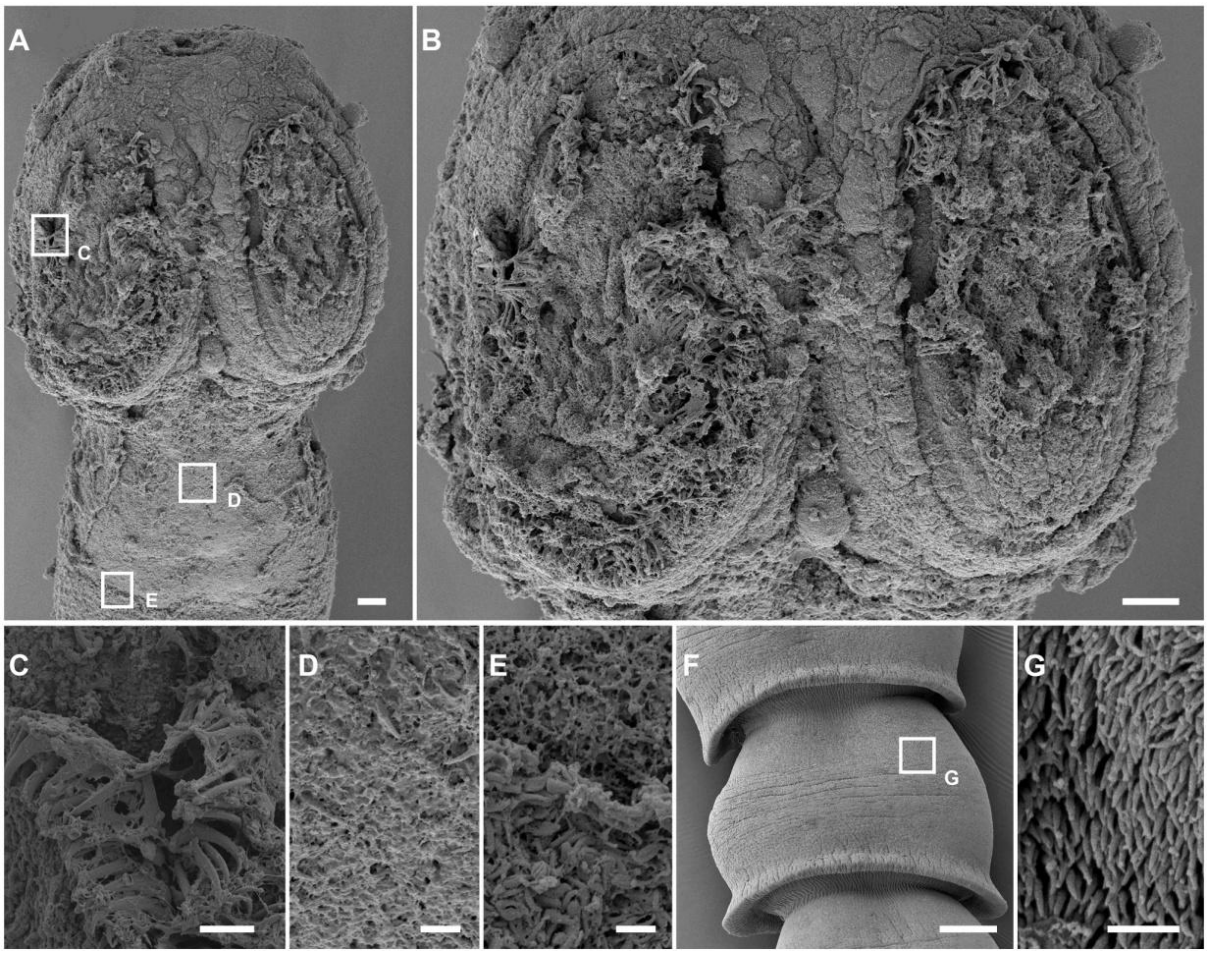

Figure S5. Scanning electron micrographs of *Raillietina* spp. treated with SQDG (1 mg/ml): (A) Scolex and neck region; note damaged suckers where hooklets were mostly broken and fallen off, and shredded microtriches (scale bar =10 μm). (B) Enlarged view of suckers; note almost no hooklets and microtriches (scale bar =10 μm). (C) Enlarged view of broken hooklets (scale bar =5 μm). (D) Enlarged view of a portion of neck region; note all microtriches were torn off (scale bar =1 μm). (E) Enlarged view of a portion of neck region; note shredded microtriches (scale bar =1 μm). (F) Mature proglottid (scale bar =100 μm). (D) Enlarged view of a portion of proglottid; note unchanged small gladiate spinitriches (scale bar =1 μm).

Reference:

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