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New Scanning Electron Microscopy Look of *Ascaridia galli* (Schrank, 1788) Adult Worm and its Biological Control

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Abstract: The *in vitro* activity of three plant extracts; *Artemisia cina, Peganum hormala* and *Calendula micrantha* was investigated against *Ascaridia galli* adult worms. *In vitro* treatment of *A. galli* worms with different concentrations of each extract revealed potency of *C. micrantha* over the other two extracts as judged by the calculated LD_{50} values. These values were 2.66 ppm for *C. micrantha*, 33.7 ppm for *P. harmala* and 48.98 ppm for *A. cina*. The impact of the most potent extract, *C. micrantha* on *A. galli* worms was detected by scanning electron microscopy. *C. micrantha* treated worms showed enlargement and destruction of lips with subsequent damage of buccal cavity organelles. Moreover, papillae were disturbed and the amphids structures were lost. The cuticle showed wrinkled surface with loss of striations. Also, the cloacal structures were affected.

Key words: Plant extracts, *Ascaridia galli*, electron microscopy

INTRODUCTION

Aacaridia galli is one of the most common parasitic round worms of poultry, occurs in chickens and turkeys. Adult worms are semitransparent and considered the biggest nematode in poultry. Heavily infected birds may show droopiness, emaciation and diarrhea. The primary damage reduced efficiency of feed utilization, but death has been observed in severe infections. Control of *A. galli* infection can be conducted through implementation of management practice and treatment strategies. The management practice includes regular cleaning and use of disinfectants, proper disposal of the used litter and farm garbage, alternate use of pens, etc. Chemotherapy is the wide use approach to control the infection. Compared to the other non-chemotherapeutic approaches to parasitic control, use of nematode trapping fungi has shown promising results in different countries (De and Sanyal, 2009). Verma *et al.* (1991) studied the comparative efficacy of three broad-spectrum anthelmintic chemotherapy against *A. galli* in poultry. Although, ivermectin proved potency against *A. galli*, as observed by Sharma *et al.* (1990), its use still limited due to its parenteral administration. Moreover, difficulties associated with chemotherapy of parasitic diseases as the ineffectiveness of some drugs, incomplete elimination of certain diseases in addition to undesired side effects looked a problem. It is not surprising therefore, to make a stress on the importance of biological control of helminth infections. Larsen *et al.* (1997), Abdel-Rahman and Saleh (2006) and Behnke *et al.* (2008) stated that biological control has the potential of

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becoming an important part of a future integrated control strategy against parasitic nematodes. The bioactivity directed fraction (Pinocembrine) of the acetone extract of *Teloxys graveolens* exhibited ovicide activity on infective eggs of *A. galli* (Comacho *et al*., 1991).

The antiascarid activity of *Caesalpinia crista* Linn seeds popularly known as Karanjwa was evaluated in chickens of the Fayomi breed, suffering from experimental infection with *A. galli* 50 mg kg^{-1} seed powder showed significant results based on excreted eggs/g faeces (Javed *et al*., 1994). The *in vitro* evaluation of the activity of root-tuber-peel extract of *Flemigia vestita*, an indigenous plant consumed by the natives in Northeast India against *Heterakis gallinarum, Raillietina echinobothrida* and *Paramphistomum* sp. was performed by Tandon *et al*. (1997). But unfortunately, only nematodes did not show any structural alternations in their tegumental architecture after exposure to *F. vestita* extract.

There are no available literatures dealing with the ultrastructure of Nematode parasite *A. galli* except Ashour (1994), who studied the scanning electron microscopy of the mentioned nematode. He described the lips, papillae, cuticle and cloaca with the prominent characteristic features for each part.

Fagerholm *et al*. (1998-2000) studied the scanning electron microscopy of *Ascaris suum* (Nematode, ascaridaidea). They described the cuticular annuli and splitting it into two subannuli during growth of the third-stage larva into adult worms. Also they stated that the increase in length of *A. suum* is due to lengthening of individual transverse annuli in the cuticle. They added that the increase in size also results in increased diameter of different cuticular sense organs. The researchers identified a two prominent asymmetrically placed cuticular sensilla, called centrids, are reported in *A. suum*. In adult worms, the centrids are plate like, asymmetrically placed. The name centrids was originally chosen to indicate the placement of the papillae in the midbody region of worms.

Based on the limited research concerned with the control of *A. galli* by plant extracts and also on the medical importance of this helminth in poultry production, the objective of the study was to focus on the *in vitro* assessment of the potency of three plant extracts in the control of *A. galli* worms utilizing scanning electron microscopy. The selection of these plants *A. cina, P. harmala* and *C. micrantha* was attributed to their previous anthelmintic impact on other helminthes (Mehrota *et al*., 1990; Hassanain *et al*., 1991; Kang, 1994; Miraldi *et al*., 1998; Shuhua *et al*., 2000; El-Garhy and Mahmoud, 2002).

MATERIALS AND METHODS

This research project was conducted from 5-2007 to 5/2010 (3 years duration) and it is carried out at the Zoonotic Diseases Department, National Research Center, Cairo, Egypt.

Collection of *A. galli* Worms

Worms were collected from the small intestine of slaughtered domestic chickens during the year of 2008. Worms were washed thoroughly with physiological saline and subjected to the *in vitro* effect of the plant extracts.

Preparation of Plant Extracts

Preparation of *A. cina* Aqueous Extract

Artemisia cina was soaked in water for 4 days at room temperature with a ratio of 1:6 weights/volume. The supernatant was filtered and lyophilized.

Preparation of *P. harmala* Ethanolic Extract

Crushed *P. harmala* seeds were soaked in ethyl alcohol for two days at room temperature with a ratio of 1: 4 weights/volume. The suspension was filtered and dried.

Preparation of *C. micrantha* Ethanolic Extract

C. micrantha flowers were soaked in ethyl alcohol at room temperature for two days at a ratio of 1: 10 weight/volume. After filtration, the clear supernatant was dried.

Treatment of *A. galli* Worms with Plant Extracts

Three concentrations (80, 160 and 320 ppm) of *A. cina*, six concentrations (5, 10, 20 , 40, 80 and 160 ppm) of *P. harmala* and five concentrations (0.5, 1, 1.5, 2 and 4 ppm) of *C. micrantha* in physiological saline were added separately to three replicates each 10 worms each. Worms were mixed and incubated at 37°C for 24 h. At the same time control untreated replicates of worms were incubated in physiological saline for 24 h. Results of worm mortality percentages were subjected to probate statistical analysis based on the work of Polo-Pc (Robertson *et al*., 1980) to determine the LD_{50} values.

Scanning Electron Microscopic Examination (SEM)

Worms treated with 4 ppm of *C. micrantha* and with the LC50 value 2.66 was prepared for scanning electron microscopy as described by Ashour and Lewis (1982). Specimens of *A. galli* were fixed in 2.5% glutraldehyde after washing. The specimens were dehydrated through ascending ethanol series, dried in CO_2 critical point drier after which they were sputter coated with gold at 20 nm. Finally, the specimens were examined and photomicrographed with SEM (Jeol, JEM-1200 EX II, Japan).

RESULTS AND DISCUSSION

Scanning Electron Microscopy of *A. galli* Adult Worms

Mouth opening of the worm was surrounded by three main large lips; one dorsal and two subventral. Each lip was not globular but it had a wide base with gentle tapering towards apex. Each main lip was provided with two small accessory ones at lateral sides. Three paired cephalic papillae and two amphids were found on the outer surface of the lips. The amphidial surface was provided with several pores. Small papillae were seen scattered on the cervical area of the worm (Fig. 1a, b).

The cuticular surface of the body was transversely striated (0.014 mm wide). These transverse annuli were well developed and appear darker in colour than the subannuli. In some sites, two successive transverse annuli might be fused at a short distance from lateral toward the middle of the cuticle with subsequent increasing the subannuli numbers and the width (0.021 mm) (Fig. 2).

Also, there were small scattered papillae with irregular pattern distribution (Fig. 1, 2). A narrow several discontinued median plate like centrids deeper to the cuticular transverse annuli extended through the whole length of the worm except the posterior one (Fig. 3, 4).

The posterior extremity of the male was slightly inflated forming ill developed caudal alae with normal cloacal protrusion (Fig. 5, 6). The ventral sucker was situated a short distance anterior to the cloacal opening, it had an ill boundered circular rim. The cloacal opening was situated on the top of a ventral conical protrusion (Fig. 5-7). The cloacal papillae were arranged in two groups, the precloacal and postcloacal ones. The precloacals papillae were arranged in the two sided-laterals of the worm. The postcloacals papillae were arranged in

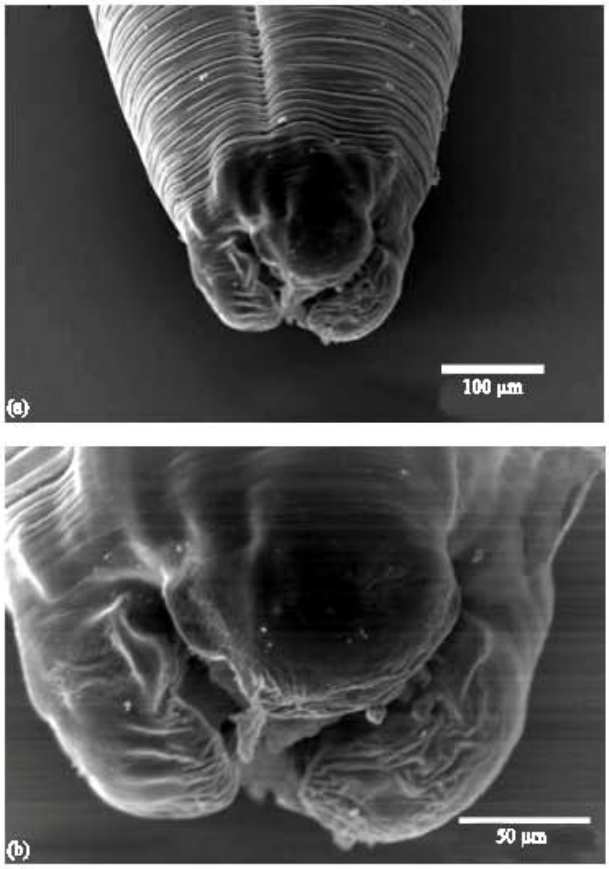

Fig. 1: (a, b) Anteroposterior view showing the mouth opening of the worm surrounded by three main large lips; one dorsal and two subventral. Each lip is not globular but it has a wide base with gentle tapering towards apex. Each main lip is provided with two small accessory ones at lateral sides. Three paired cephalic papillae and two amphids are found on the outer surface of the lips. The amphidial surface is provided with several pores. Small papillae were seen scattered on the cervical area of the worm

Table 1: *In vitro* anthelmintic effect of the plant extracts on *galli* worms

Plant extract	Conc. (mg)	Worm mortality	LC_{50} value (mg)
Artemisia cina	80.0	60.0	48.98
	160.0	66.7	
	320.0	80.0	
Peganum harmala	5.0	0.0	33.70
	10.0	20.0	
	20.0	33.3	
	40.0	73.3	
	80.0	93.3	
	160.0	100.0	
Calendula micrantha officinalis	0.5	0.0	2.66
	1.0	6.6	
	1.5	13.3	
	2.0	26.7	
	4.0	66.6	

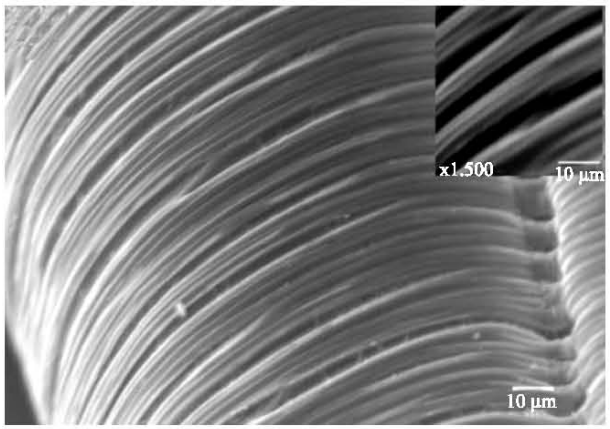

Fig. 2: Anteroposterior view showing the cuticular surface of the body which is transversely striated (0.014 mm wide). These transverse annuli are well developed and appear darker in colour than the subannuli. In some sites, two successive transverse annuli may be fused at a short distance from lateral toward the middle of the cuticle with subsequent increasing the subannuli numbers and the width (0.021 mm)

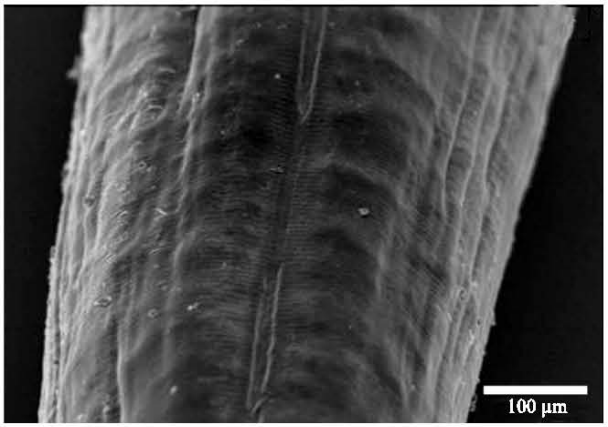

Fig. 3: Anteroposterior view showing small scattered papillae with irregular pattern distribution. Several narrow median centrids

one group. The number of the cloacal papillae was several and could not be counted (Fig. 6, 7).

The *in vitro* impact of the three plant extracts on *A. galli* worms was depicted on Table 1 proving the advantage of *C. micrantha* over the other extracts. The impact was assessed by calculating the LD_{50} which was 48.98 ppm for *A. cina* extract, 33.7 ppm for *P. harmala* and 2.66 ppm for *C. micrantha*.

The worms group that showed high mortality rate and potent LC50 was selected to be subjected to SEM directly.

In vitro Effect of *C. micrantha* Extract as Anthelmintics against *A. galli* Adult Worms: The three lips guarded the mouth opening of the worm were greatly affected together with the buccal cavity. There were morphological disfigurements of the three lips (one dorsal and

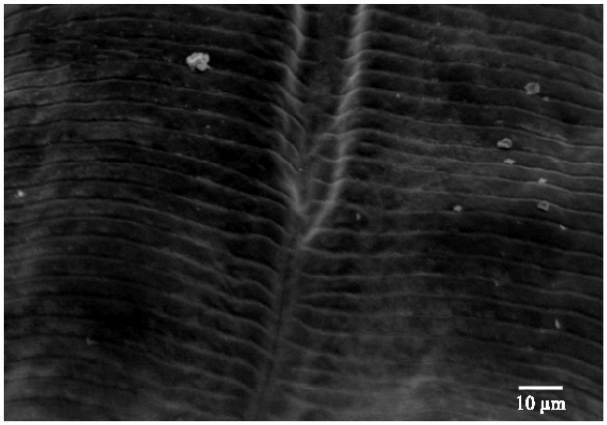

Fig. 4: Anteroposterior view showing several narrow discontinued median centrids deeper to the cuticular transverse annuli which, extend through the whole length of the worm except the posterior one

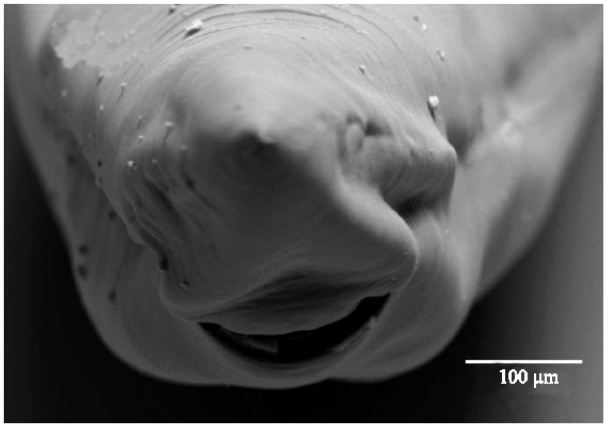

Fig. 5: Ventral view showing the posterior extremity of the male and the ventral sucker is situated a short distance anterior to the cloacal opening, it has an ill boundered circular rim

two subventral ones). The lips became globular or quadrangular and the smaller accessory lips were separated and lost from the two subventral large lips. But in the dorsal one, was partially separated and still connected to the mother one (Fig. 8, 9). The mouth opening and cavity were occluded by granulation tissues in the form of triangular plate with adhesion to the surrounding lips which lead to widening of the lips (Fig. 8, 9). Also, the amphids architectures were lost due to the compressed lips (Fig. 8, 9). The cuticular striations were lost due to its swelling with longitudinal or transverse dividing masses (Fig. 8, 9).

Concerning the cloacal area, the precloacal papillae distribution were lost and the ventral sucker lost its demarcation with area of hemorrhage together with the surrounding tissues and the cloacal opening was still patent opened with intact edges (Fig. 10a, b). The cloacal protrusion was severely destructed and its tip was protruded to outside with herniation of its internal content (Fig. 11).

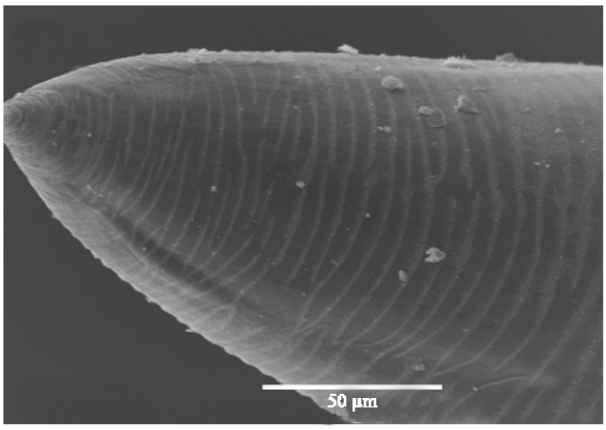

Fig. 6: Lateral view showing the posterior extremity of the male which is slightly inflated forming ill developed candal alae with normal cloacal protrusion. The cloacal opening is situated on the top of a ventral conical protrusion, the cloacal papillae that are arranged in two groups, the precloacal and postcloacal ones

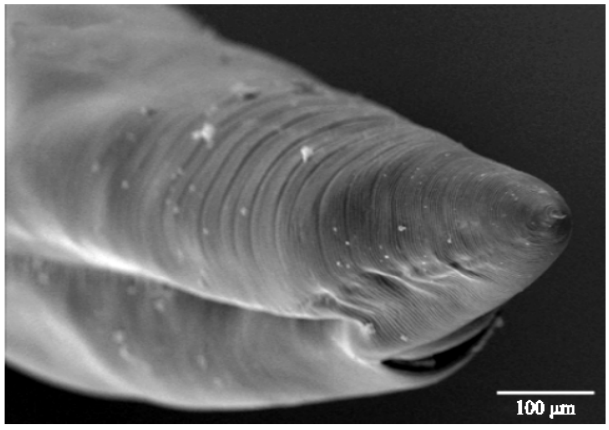

Fig. 7: Lateral view showing the cloacal papillae which are arranged in two groups, the precloacal and postcloacal ones. The precloacals papillae are arranged in the two sided-laterals of the worm. The postcloacals papillae are arranged in one group

The *in vitro* study, performed in the current research, revealed the potent lethal effect of *C. micrantha* extract against *A. galli* adult worm recording LD_{50} value of 2.66 ppm. While *P. harmala* showed lesser effect with LD_{50} of 33.7 ppm followed by *A. cina* with LD_{50} of 48.98 ppm. The potent lethal effect of *C. micrantha* against nematodes was previously recorded against larvae of *Haomonch contortus*, *Trichostrongylus colubriformis, Bunostomum* spp. and *Toxocara vitulorum* eggs as observed by Hassanain *et al*. (1991). In contrary to the recorded weak effect of *A. cina* observed in the present study, *A. santonica* proved ascaridial efficacy *in vitro* against eggs and larvae of *Ascaris lumbricoides* (El-Garhy and Mahmoud, 2002). The difference between both observations could be attributed to the difference of the species of the used plant and to the developmental stages of both worms.

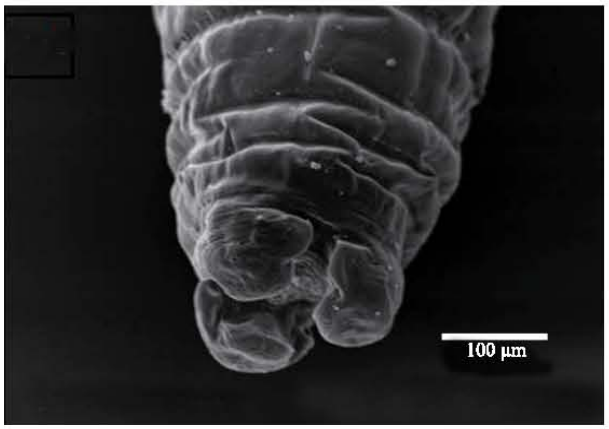

Fig. 8: Anteroposterior view showing the great affection of three lips guarded the mouth opening of the worm together with the buccal cavity. There are morphological disFig. ments of the three lips (one dorsal and two subventral ones). The lips became globular or quadrangular and the smaller accessory lips were separated and lost from the two subventral large lips. The dorsal one is separated and still connected to the mother one

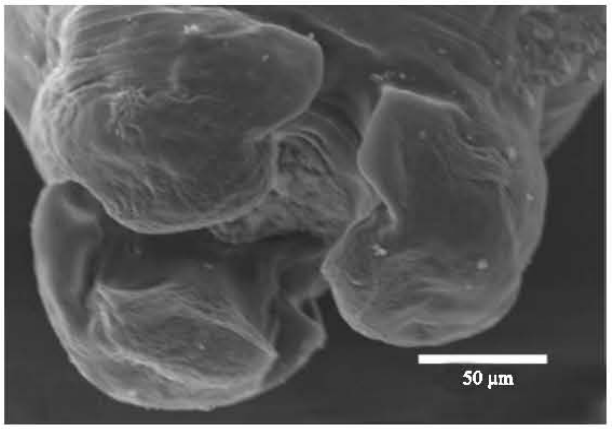

Fig. 9: Anteroposterior view showing the mouth opening and cavity occluded by granulation tissues in the form of triangular plate with adhesion to the surrounding lips which lead to widening of the lips. Also the amphids architectures were lost due to the compressed lips. The cuticular striations were lost due to its swelling with longitudinal or transverse dividing masses

Scanning electron microscopical examination was used to assess the effect of *C. micrantha*, the most potent extract against *A. galli* worm compared with normal structure. Scanning electron microscopy of normal *A. galli* was previously described by Ashour (1994) except in some detailed morphological features as observed in the current research. Ashour (1994) stated that the lips of the *A. galli* worm has a wide base and narrow extremity with trilobed appearance and the inner surface of each lip is provided with a thick smooth cuticular plate. The cuticular surface of the body is transversely striated with smaller diameter

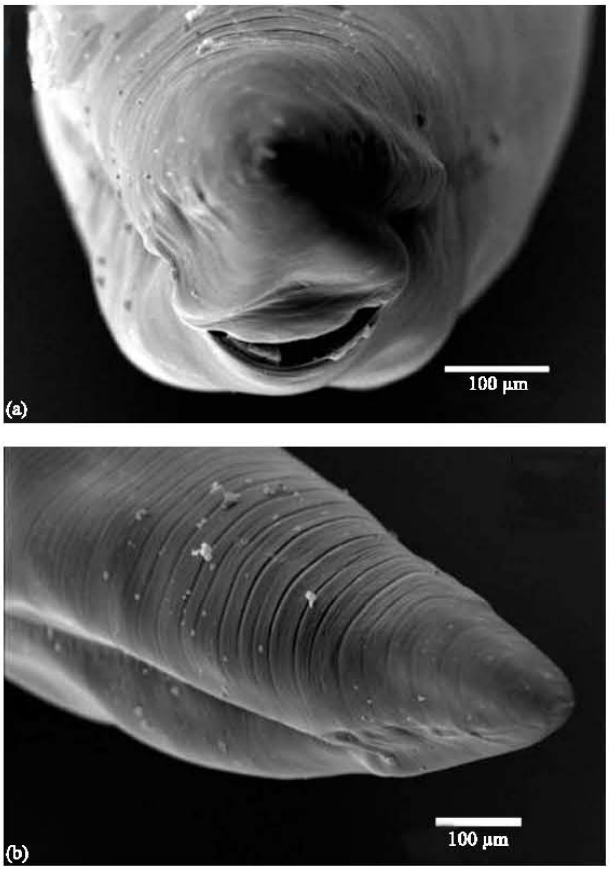

Fig. 10: (a, b) Face view showing the loss of precloacal papillae distribution and the ventral sucker loss its demarcation with area of hemorrhage together with the surrounding tissues and the cloacal opening is still patent opened with intact edges

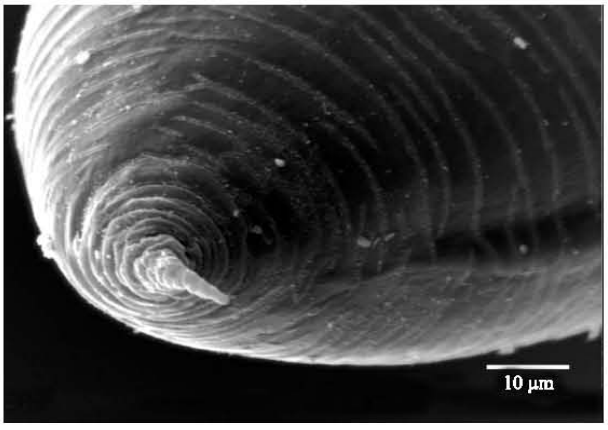

Fig. 11: Face view showing the severe destruction of cloacal protrusion and its tip is protruded to outside with herniation of its internal content

(0.006 mm wide) than proved in the present study (0.014 mm wide). In addition no subannuli were detected in his study, while the subannuli were observed, for the first time, in the current research. The cloacal papillae are arranged as follows: 3 pairs of precloacales, 2 pairs of lateral and 2 pairs of ventral postcloacal and one pair of lateral and one pair of ventral subterminal papillae. This pattern of distribution is different from that currently described. For the first time, the present study revealed new morphological characteristics in *A. galli* as those shown by Fagerholm *et al.* (1998-2000) and Fagerholm *et al.* (1999) in scanning electron microscopy of *A. suum*. There are circular or transverse annuli which were split into subannuuli during embryonic development of *A. galli* larvae to adult and also it's lengthening in size to adult due to the cuticular development of worm. The presence of centrids in *A. galli*, which is described currently for the first time, is very important and aids in its morphological discrimination from *A. suum*.

Treatment of *A. galli* worms with *C. micrantha* resulted in dramatic changes in the mouth parts and cuticle. The changes in the mouth parts are associated mainly with the lips which showed enlargement and damage of the sensory papillae. These changes unable the worms to fix themselves to intestinal mucosa and led to their expulsion. Consequently, it may be postulated that, the mode of action of *C. micrantha* plant extract is worm fuge. On the other hand, the effect of both plants on the cuticular structure probably affects its function such as nutritional uptake which resulted in worm mortality and in this case may be act as wormicide. Unfortunately, no literatures are available regarding the assessment of the impact of plant extracts on *A. galli* worm using scanning electron microscopy which could support our postulations or not.

CONCLUSION

The present study introduced a biological control model of *A. galli* adult worms, *in vitro*, using plant extracts and clarified, at the level of ultra structural examination, the damages associated with worm organelles.

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