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Body Surface Changes in Gastrointestinal Helminthes Following *in vitro* Treatment with *Allium sativum* Oil

Hatem A Shalaby* and Tarek K Farag

Department of Parasitology and Animal Diseases, National Research Center, Giza, Egypt

Abstract

A scanning electron microscopic study was undertaken to assess, for the first time, whether *Allium sativum* oil (ASO) extract had any effect on helminthes' body surface following 24 h incubation *in vitro*. Two species of gastrointestinal helminthes, of most economic significance in sheep, *Haemonchus contortus* and *Moniezia expansa* were collected from naturally infected sheep slaughtered in Cairo abattoir, then exposed to 10, 50, 100 µg/ml ASO. The body surface of these helminthes was observed to be affected and altered by ASO. These changes were concentration dependent, and consisted of destructive alterations and deformity in the cuticle of *H. contortus* and also in the tegumental architecture of *M. expansa*. The current study confirmed that an alternative, effective and natural anthelmintic could be developed using garlic.

Keywords: *Haemonchus contortus*; *Moniezia expansa*; *Allium sativum* oil; *In vitro*

Introduction

Gastrointestinal helminthes are the highest disease cost to small ruminants industry. The costs associated with parasite infection are categorised as direct and in-direct. The direct cost is the cost of treatment such as the anthelmintic, labour and animal deaths. The in-direct cost of parasitism is the loss of productivity such as reduced growth and live weight of infected animals, reduced milk production and reductions in wool growth and quality [1]. Small ruminants have numerous internal parasites; two of the most economic significance are the nematode *Haemonchus contortus* [2] and the cestode *Moniezia expansa* [3]. These two parasites have more reported cases of anthelmintic resistance around the world than other gastrointestinal parasites as well their prevalence and effects on the host [4]. The development of parasite anthelmintic resistance has been influenced by producers' over-reliance on these chemicals to control parasites as well as poor management practices such as under-dosing [5]. With the increased occurrence of multiple drug resistant parasites, other more sustainable methods are being investigated, including the use of medicinal plant extracts with anthelmintic properties, such as garlic.

Garlic (*Allium sativum*) is one of the oldest, most common medicinal herbs still used today. It has been reported to be a parasiticide, amebicide, acarifuge, vermifuge, larvicide, fungicide, and immuno-stimulant besides other properties [6]. It has been used to treat animals that suffer from gastrointestinal parasitism [7]. Garlic oil has a broad-antimicrobial spectrum; as it has antibacterial, antifungal, antiviral, and antiparasitic effects. Further, it influences the growth of at least 12 different human and nonhuman parasites [8].

The external surfaces of helminths serve as a barrier that shields the organism from external conditions. These surfaces are also vital for nutrient uptake, osmoregulation, immunoprotection and structural support [9]. Since the integrity of the external surfaces of helminths is essential for the nutritive and protective functions, scanning electron microscopic study was undertaken to assess, for the first time, whether the garlic oil had any effect on the cuticle of *H. contortus* and the tegument of *M. expansa* following incubation *in vitro*.

Materials and Methods

Preparation of *A. sativum* oil extract

Bulbs of fully grown *A. sativum* cultivated locally were used in the study. The dried bulbs were pounded and then steam distilled. The essential oil was extracted using solvent ether.

Anthelmintic effects of *A. sativum* oil

Adult worms of *H. contortus* (nematode) and *M. expansa* (cestode) were collected from abomasum and intestine, respectively, of naturally infected sheep slaughtered in a local abattoir in Cairo province, Egypt. After recovery, the worms were washed and transferred to Ringer solution, as recommended by Hanser et al. [10] containing ASO at concentrations of 10, 50 and 100 µg/ml. The worms were incubated for 24 h at 37°C in an atmosphere of 5% CO_2. The ASO was initially prepared as a stock solution in DMSO and added to the culture medium to give a final solvent concentration of 0.01% (v/v). Solvent control worms were incubated for 24 h in Ringer solution containing 0.01% (v/v) DMSO. Fresh control worms at 0 h were fixed immediately following the initial washing. Five worms were examined for each group.

Scanning Electron Microscopy (SEM)

Following incubation, the anterior end of adult worms was fixed intact for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12 M-Millonig's buffer, pH 7.4 and 1% aqueous osmium tetroxide. Then specimens were processed for SEM following a method previously reported [11].

***Corresponding author:** Hatem A Shalaby, Department of Parasitology and Animal Diseases, National Research Center, Egypt, Tel: 020 3371615; Fax: 020 3370931; E-mail: shalaby85@gmail.com

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Results

Scanning electron microscopic observations of the anterior end of adult *H. contortus*

Normal freshl worms

The mouth of the normal fresh worm was hexagonal with six semicircular rudimentary lips, lateral amphids and papillae. A pair of cervical papillae was prominent and spine-like (Figure 1a). The cuticle was transversally striated and with lateral ridges (Figure 1b).

Treated worms

After 24 h incubation with 10 µg/ml ASO, the cuticle appeared to be more swollen than normal so that the transverse striations became less pronounced and lost their normal aspect showing longitudinal wrinkles (Figure 1c and 1d). The cuticular swelling became pronounced

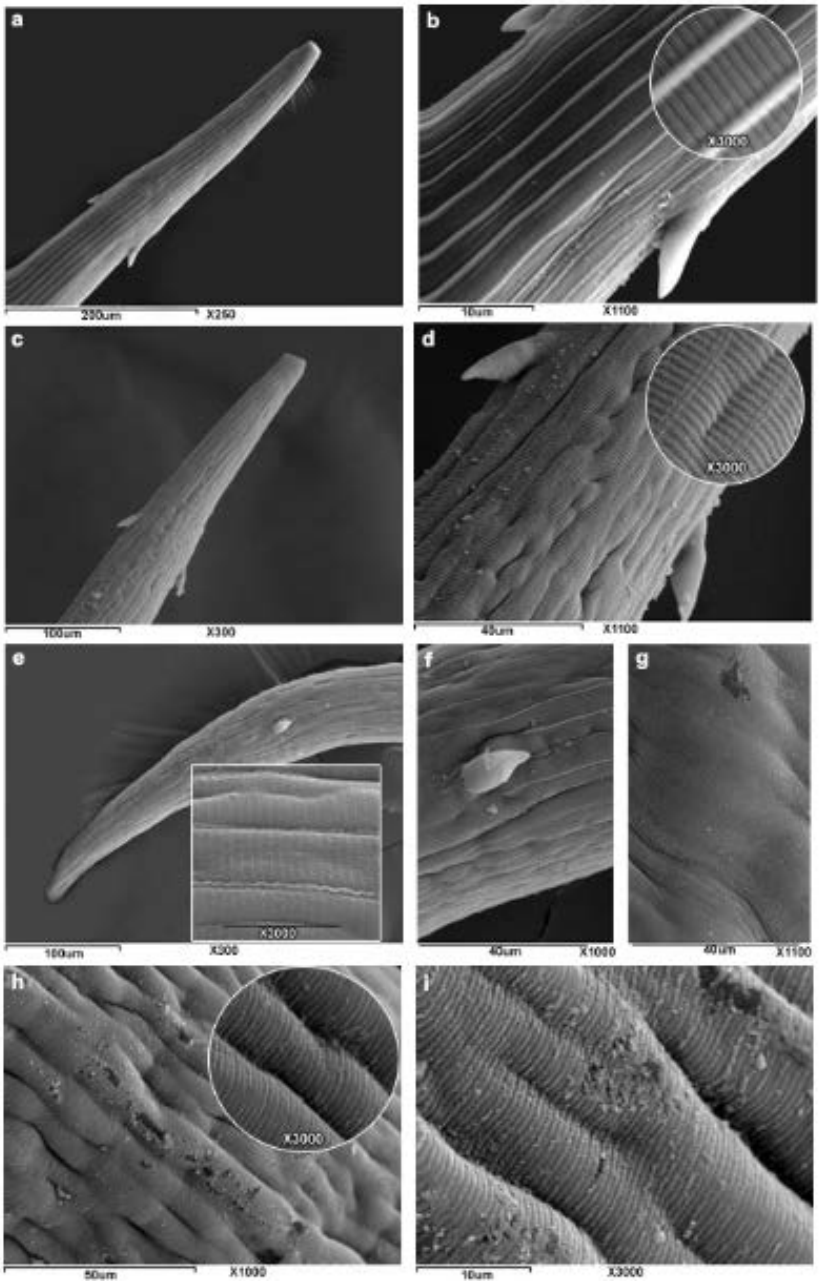

Figure 1: Scanning electron micrographs (SEMs) of the anterior end of adult *Haemonchus contortus*.

(a, b) SEMs of normal fresh worm showing the mouth with six semicircular rudimentary lips and a pair of spine-like cervical papillae. The cuticle is transversally striated and with lateral ridges (Inset). **(c, d)** Following 24 h of incubation with 10 µg/ml ASO, showing slightly more swollen cuticle than normal with less pronounced transverse striations (Inset). **(e-g)** Following 24 h of incubation with 50 µg/ml ASO, revealing wrinkled and corrugated cuticular surface with ill distinguished cuticular striations. **(h, i)** Following 24 h of incubation with 100 µg/ml ASO, showing severely folded and corrugated cuticle (Inset) and small areas of lesions scattered over its surface.

with wrinkling of the cuticular ridges, on increasing the concentration to 50 µg/ml ASO (Figure 1e). Besides, the cuticular surface had a wrinkled, corrugated appearance (Figure 1f). In extreme cases, the cuticular swelling became so severe so that the cuticular striations were ill distinguished (Figure 1g). With higher concentration of 100 µg/ml ASO, the distortion of the cuticle was similar to that described for the previous concentration, except it was more extensive. But what was apparent in this region was the presence of small areas of lesions scattered over the cuticular surface (Figure 1h and 1i).

Scanning electron microscopic observations of the anterior end of adult *M. expansa*

Normal fresh worms

The scolex appeared globular and was provided with four oval suckers radially located around the proximal end of the scolex (Figure 2a). The strobila was an elongated ribbon-like structure composed of series of segments (proglottids) which were broader than longer (Figure 2b).

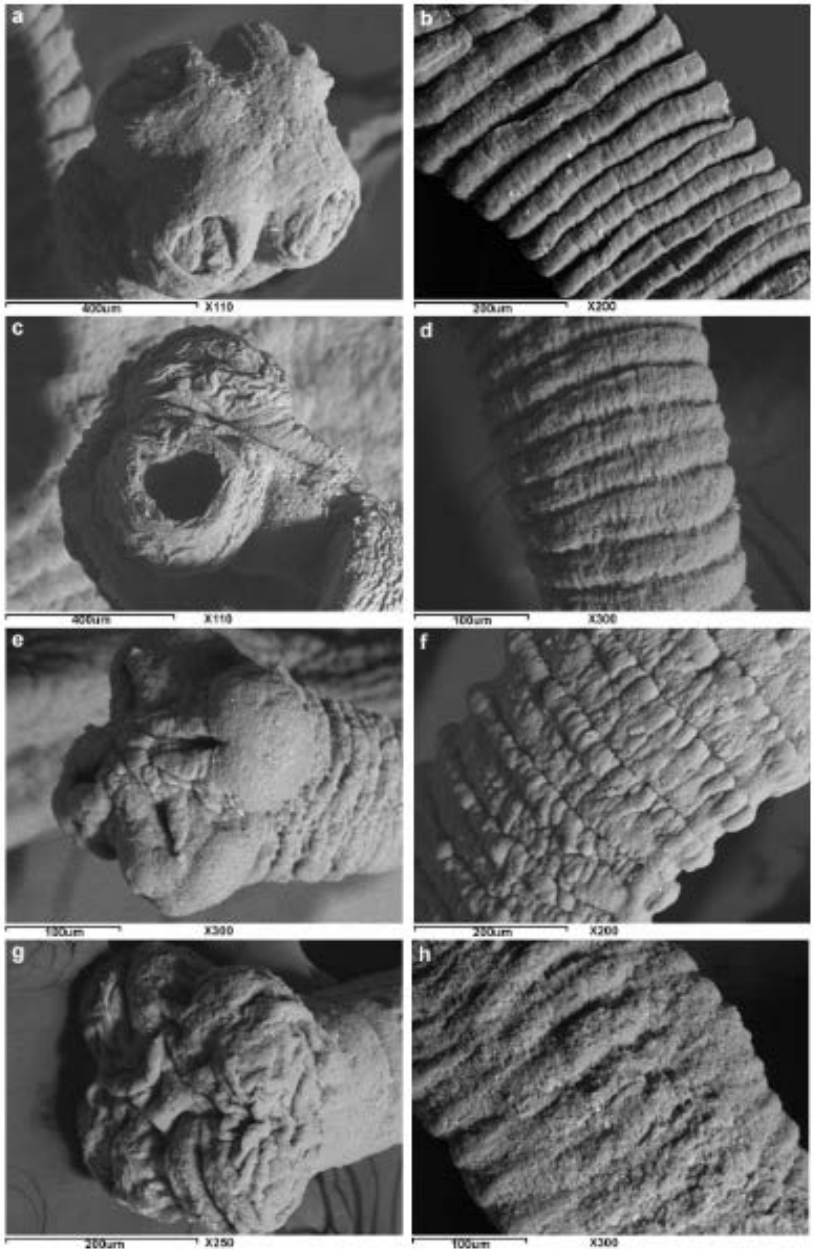

Figure 2: SEMs of the anterior end of adult *Moniezia expansa.*

(a, b) SEMs of normal fresh worm showing a globular scolex with four oval suckers and an elongated ribbon-like structure called strobila. **(c, d)** Following 24 h of incubation with 10 µg/ml ASO, showing wrinkled tegumental surface throughout the strobila. **(e, f)** Following 24 h of incubation with 50 µg/ml ASO. The scolex becomes more swollen than normal with narrowing of sucker's opening. The proglottides appear deformed with circular areas of pronounced swelling occurred along their margins. **(g, h)** Following 24 h of incubation with 100 µg/ml ASO, showing shrunken scolex with severely distorted and folded tegument.

Treated worms

After 24 h incubation with 10 µg/ml ASO, the changes in adult cestodes concerned the proglottides other than the scolex which retained its normal morphology (Figure 2c). The proglottides lost their normal aspect showing wrinkled tegumental surface throughout the strobila (Figure 2d). On increasing the concentration to 50 µg/ml ASO, the changes concerned the whole-body surface.The scolex appeared to be more swollen than normal with contractions to the sucker's opening (Figure 2e). The proglottides appeared deformed with circular areas of pronounced swelling occurred along their margins (Figure 2f). With higher concentration of 100 µg/ml ASO, the adult cestodes showed shrunken scolex with severely distorted and folded tegument around the suckers so that a complete closure of their openings had occurred (Figure 2g). In the proglottides, the tegument was swollen and severely distorted (Figure 2h).

In all of the experiments, no significant differences were observed between fresh and control worms incubated for 24 h in solvent, 0.1% (*v*/*v*) DMSO.

Discussion

The present study demonstrated the effect of ASO on gastrointestinal helminthes. Scanning electron microscopic observations could be used to determine, for the first time, the target of ASO as morphological changes could be observed. These changes were concentration dependent, and consisted of destructive alterations and deformity in the cuticle of *H. contortus* and also in the tegumental architecture of *M. expansa*. The cuticle of nematodes is metabolically active and morphologically specialized for selective absorption of nutrients and osmoregulation. Thus, passive diffusion of anthelmintics through the cuticle [9] would probably be responsible for destructive changes and deformation of the nematode body surface [12]. In cestodes, the general body surface acts as an absorptive surface [3]. In the present investigation, the body surface of these helminthes was observed to be affected and altered by ASO. Similar to the present observations, the surface cuticle or tegument was found to be a principal target site for different synthetic drugs and natural anthelmintic products as proved by histomorphological and ultrastructural studies [4,11,13,14]. The swelling and distortion of the body surface of *H. contortus* and *M. expansa* induced by ASO had been described for these helminthes by *Nigella sativa* oil [4]. In *Fasciola hepatica*, a similar pattern of tegumental swelling had been described following incubation with a number of anthelmintics, as summarized by Hegazi et al. [15]. This was a common feature of drug treated parasites and considered to be an indicator of a stress reaction by the parasite, in an attempt to replace damaged surface membrane [16].

The literature survey of *A. sativum* suggested that it had anthelmintic action *in vitro* against *H. contortus* [17], *Heterakis gallinae* and *Ascaridia galli* [18], a free-living nematode of *Rhabditis* sp, larvae of *Nippostrongylus brasiliensis,* and eggs of *Ascaris sum* [19]. Besides, it had antiprotozoal [20] and acaricidal [21] effects. The bioactive property of garlic was formed by the interaction of the non-protein amino acid alliin and the enzyme alliinase [22]. Thiosulfinates were produced through interaction, most commonly induced by crushing the mature garlic clove, of which around 70% of the bioactive compounds produced was allicin (diallylthiosulphinate) [22,23]. Allicin was responsible for garlic's pungent odour and the many medicinal health benefits associated with the consumption of garlic [24]. Garlic essential oil was produced by grinding whole garlic cloves in water; the oil fraction was then obtained by either heat distilling or extraction with an organic solvent. Seventy to eighty percent of the thiosulphinates produced in this oil were made up of allicin (diallylthiosulfinate) [25, 26]. Garlic oil was reported to inhibit the growth of more than 12 human and non-human parasites [8]. The mode of action of allicin varied depending on the species of parasite. Williams and Lamprecht [27] and Anthony et al. [8] stated that some of actions on parasites were inhabit/blocking of receptor sites of cysteine proteinases, phosphatidylcholine biosynthesis, and the synthesis of coenzyme Q and cell lysis. Other parasitic modes of action were the interactions with thiol-containing enzymes, alcohol dehydrogenases, thioredoxin reductases, as well as the alteration of intracellular membranous structures. When allicin broke down, one of the chemicals, ajoene, was reported to be antiparasitic, which interfered with lipid and protein absorption in the parasite and resulted in a broke down in the intracellular membrane system and cell lysis [8]. There were also reports of garlic being ineffective as an anthelmintic in experimental procedures both *in vitro* and *in vivo* which could be due to the preparation methods. The failure of garlic as an anthelmintic to donkeys in the trial by Sutton and Haik [28] was attributed to an inappropriate extraction method and/or the dose rate, while in a trial by Pena et al. [29] where no extraction method was used and the garlic was fed intact there was a 100% reduction in worm burdens.

The current study and those cited above indicate that an alternative, effective and natural anthelmintic can be developed using garlic that could offer a suitable and cheaper alternative for the more expensive anthelmintics. Further studies with *in vivo* effect of ASO on gastrointestinal helminthes are clearly warranted.

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