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In vitro Efficacy of a Combination of Ivermectin and *Nigella sativa* Oil Against Helminth Parasites

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Abstract: The current study had been carried out *in vitro* to investigate the comparative morphological effects of ivermectin/*Nigella sativa* oil combination and each of them separately against adult helminth parasites include *Haemonchus contortus*, *Moniezia expansa* and *Fasciola gigantica*. The live adult parasites were collected from naturally infected sheep slaughtered in Cairo abattoir, then exposed to 50 ng/ml ivermectin, 1 mg/ml *N. sativa* oil extract and a combination of ivermectin and *N. sativa* oil in half concentration. Surface changes to the parasites were assessed by scanning electron microscopy. The results revealed that the combination of half dose of ivermectin/*N. sativa* oil was more destructive to the cuticular or tegumental surfaces of adult helminthes than each of them separately. The present study had provided morphological evidence for the greater anthelmintic activity of ivermectin on combination with *N. sativa* oil and the results lent support to the idea of using drug combinations against helminthes infections.

Key words: Ivermectin · *Nigella sativa* Oil · Combination Therapy · Helminthes · *In vitro*

INTRODUCTION

Sheep production is an attractive source of income for farmers in Egypt, due to low capital input and the ability of sheep to thrive on native pasture grasses. However, productivity is constrained by parasitic infections. Helminth infections are amongst the most common parasitic infections of animals worldwide and are now well recognized as an important veterinary problem, both in developing and in developed countries [1, 2]. The sheep industry still relies heavily on the use of anthelmintics to alleviate the infections of gastrointestinal nematodes, cestodes and liver flukes that are the most important. At a time, anthelmintic resistance has become a serious problem in veterinary medicine [3- 5]. A number of strategies have been proposed to try and preserve the efficacy of existing drugs and slow down the spread of resistance. Strategies involve the use of alternative anthelmintics, the rotation of drugs from different chemical groups, the selective treatment of animals and the use of drug combinations [6]. Combinations of two or more drugs are a routine part of parasite control in livestock. They can be used to treat mixed infections as triclabendazole (the fluke drug) is used in combinations with drugs target nematodes, such as levamisole, oxfendazole, ivermectin, or abamectin. Using anthelmintics from different chemical groupings (and with different mechanisms of action) achieve an additive or synergistic effect. With synergistic combinations, the possibility of using lower-than-normal concentrations would reduce drug costs and side-effects and reduce drug residues in host tissues and in the environment. A combination of mebendazole and levamisole has been shown to be synergistic against *Haemonchus contortus* in sheep [7]. Febantel and pyrantel showed synergistic action against *Ancylostoma caninum* in dogs [8] and *Heterakis spumosa* in mice [9]. Moreover, febendazole and pyrantel proved synergistic effect against *Toxocara canis in vitro* [10]. The present study reports on the activity of an ivermectin/*Nigella sativa* oil combination to enable treatment of both gastrointestinal helminthes and trematode infections simultaneously, since ivermectin is a very effective anthelmintic for the treatment of nematode infections [11-14] and *Nigella sativa* oil has high activity against cestodes and liver flukes [15-17].

The present study was carried out *in vitro* to examine the morphological effects of an ivermectin/*Nigella sativa* oil combination against helminth parasites; nematode, cestode and trematode.

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MATERIALS AND METHODS

Drug: Ivermectin (Iveen®) was obtained from ADWIA Company, 10th of Ramadan City, Cairo, Egypt, in the form of 10 mg/ml injection.

Preparation of *N. sativa* Oil Extract: The dried seeds of *N. sativa* were purchased from a local market and authenticated at the Herbarium of National Research Center. The seeds were crushed and cold macerated in petroleum ether (40-60°C) for three days. After evaporation of petroleum ether, the extract was taken out and the oil was filtered. The extracted oil was kept in screw-capped tubes in the dark at - 20°C until use [18].

Anthelmintic Effects of Ivermectin And/or *N. sativa* Oil: Adult worms of *Haemonchus contortus* (nematode), *Moniezia expansa* (cestode) and *Fasciola gigantica* (trematode) were collected from abomasums, intestine and bile ducts, respectively, of naturally infected sheep slaughtered in Cairo abattoir and washed in several changes of warm (37°C), sterile complete RPMI 1640 culture medium containing antibiotics (penicillin, 50 IU/ml; streptomycin, 50 mg/ml). The worms were subsequently transferred to fresh culture medium containing 50 ng/ml ivermectin; corresponded to maximum blood levels *in vivo* [19] or *N. sativa* oil extract at concentration of 1 mg/ml; the lowest concentration that could kill adult worms *in vitro* [15]or both ivermectin and *N. sativa* oil in half concentration. The *N. sativa* oil was initially prepared as a stock solution in DMSO and added to the culture medium to give a final solvent concentration of 0.03% (v/v). Other group was prepared by incubating worms in RPMI 1640 culture medium containing 0.03% (v/v) DMSO; as solvent control. The worms were incubated for 24 h at 37°C in an atmosphere of 5 % CO_2. Normal control worms at 0 h were fixed immediately following the initial washing. Six 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 [20].

RESULTS

***In vitro* Effects of Ivermectin And/or *N. sativa* Oil on Adult *H. contortus*:** *Scanning electron microscopy (SEM) of normal fresh worms*

The mouth of the normal fresh worm was hexagonal with six semicircular rudimentary lips, lateral amphids, papillae and dorsal buccal lancet (Fig.1a and inset). The later was a specialized cuticular structure arising from the cuticular lining of the dorsal wall of the buccal cavity. The lancet's anterior tip and lateral edges were slightly rounded (Fig. 1a inset). A pair of cervical papillae was prominent and spine-like (Fig. 1a). The cuticle was transversally striated and with lateral ridges (Fig. 1b).

***SEM of Treated Worms*:** The changes in *H. contortus* adult worms after 24 h incubation with 50 ng/ml ivermectin concerned the buccal capsule other than the cuticle. The buccal capsule which presented a smooth surface in control worm, lost their normal aspect and showed distortion with severe blebbing of the lips (Fig.1c and inset). No obvious differences were detected between the treated and untreated worms concerning their cuticle (Fig.1d).

After 24 h incubation with 1 mg/ml *N. sativa* oil, the cuticle including the lips appeared to be more swollen than normal so that the transverse striations became less pronounced and lost their normal aspect showing longitudinal wrinkles (Fig.1e and f).

The strongest effects were observed after 24 h incubation with drug combination (25 ng/ml ivermectin + 0.5 mg/ml *N. sativa* oil), where the treated worms showed distortion of both buccal capsule and cuticle (Fig. 1g). The lips were deformed (Fig. 1g inset) and the cuticular surface had a wrinkled, corrugated appearance (Fig. 1h). Besides, longitudinal thickening and wrinkling of the cuticular ridges were observed.

In vitro* Effects of Ivermectin And/or *N. sativa* Oil on Adult *M. expansa

***SEM of normal fresh worms*:** The scolex appeared globular and was provided with four oval suckers radially located around the proximal end of the scolex (Fig. 2a). The strobila was an elongated ribbon-like structure composed of series of segments (proglottids) which were broader than longer (Fig. 2b). Each segment contained a common genital pore on either side (Fig. 2b inset).

***SEM of Treated Worms*:** After 24 h incubation with 50 ng/ml ivermectin, the changes in adult cestodes concerned the proglottides other than the scolex which retained its normal morphology (Fig. 2c). The proglottides lost their normal aspect showing wrinkled tegumental surface throughout the strobila (Fig. 2d).

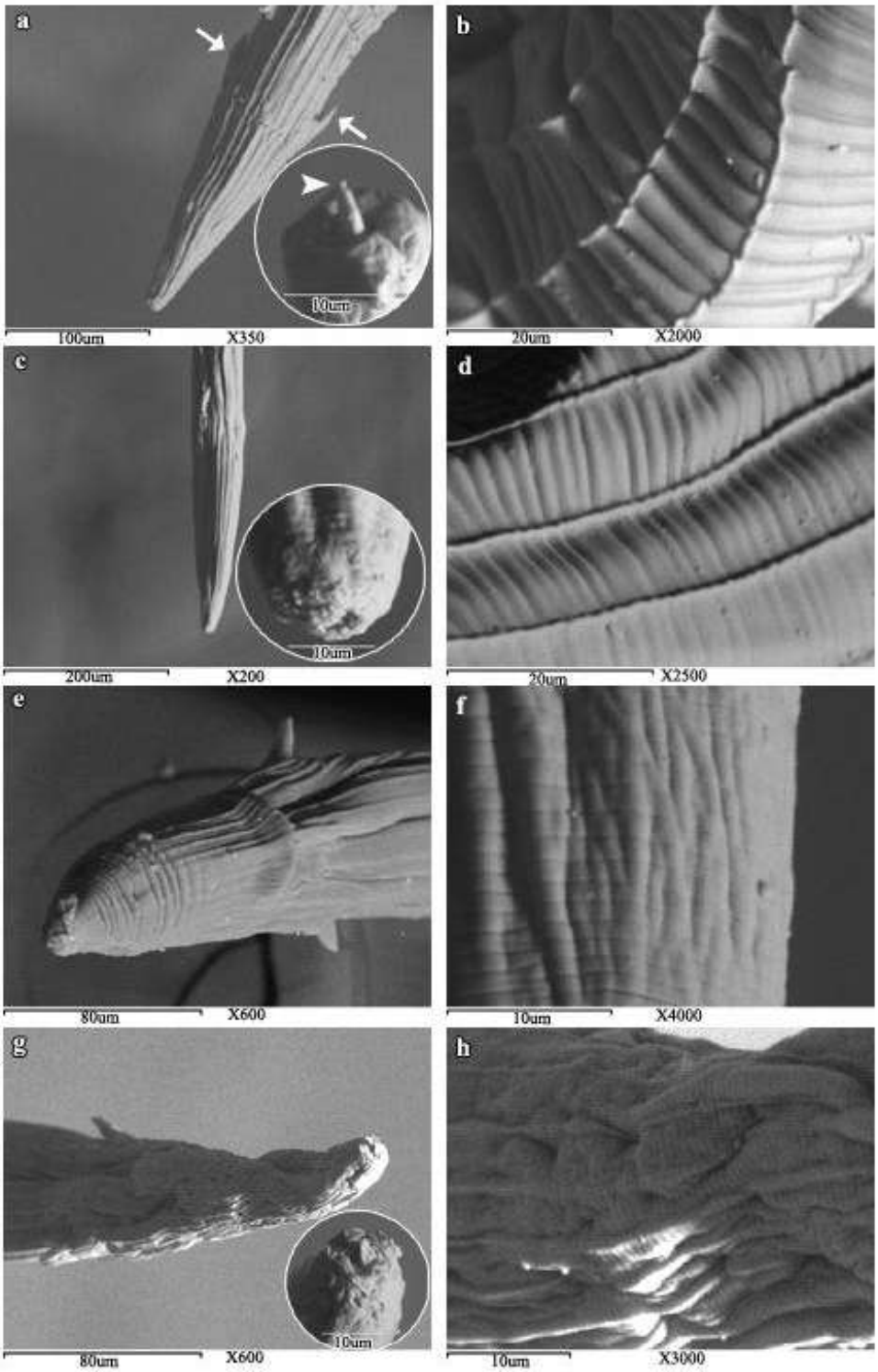

Fig. 1: Scanning electron micrographs (SEMs) of the anterior end of adult *Haemonchus contortus*. (a, b) SEMs of normal fresh worm showing the mouth with six semicircular rudimentary lips and a pair of spine-like cervical papillae (arrows). *Inset* demonstrates a dorsal buccal lancet (head arrow). (c, d) Changes after 24 h of incubation in 50 ng/ml ivermectin. (e, f) Changes after 24 h of incubation in 1 mg/ml *N. sativa* oil. (g, h) Changes after 24 h of incubation in a combination of half-strength ivermectin and half-strength *N. sativa* oil.

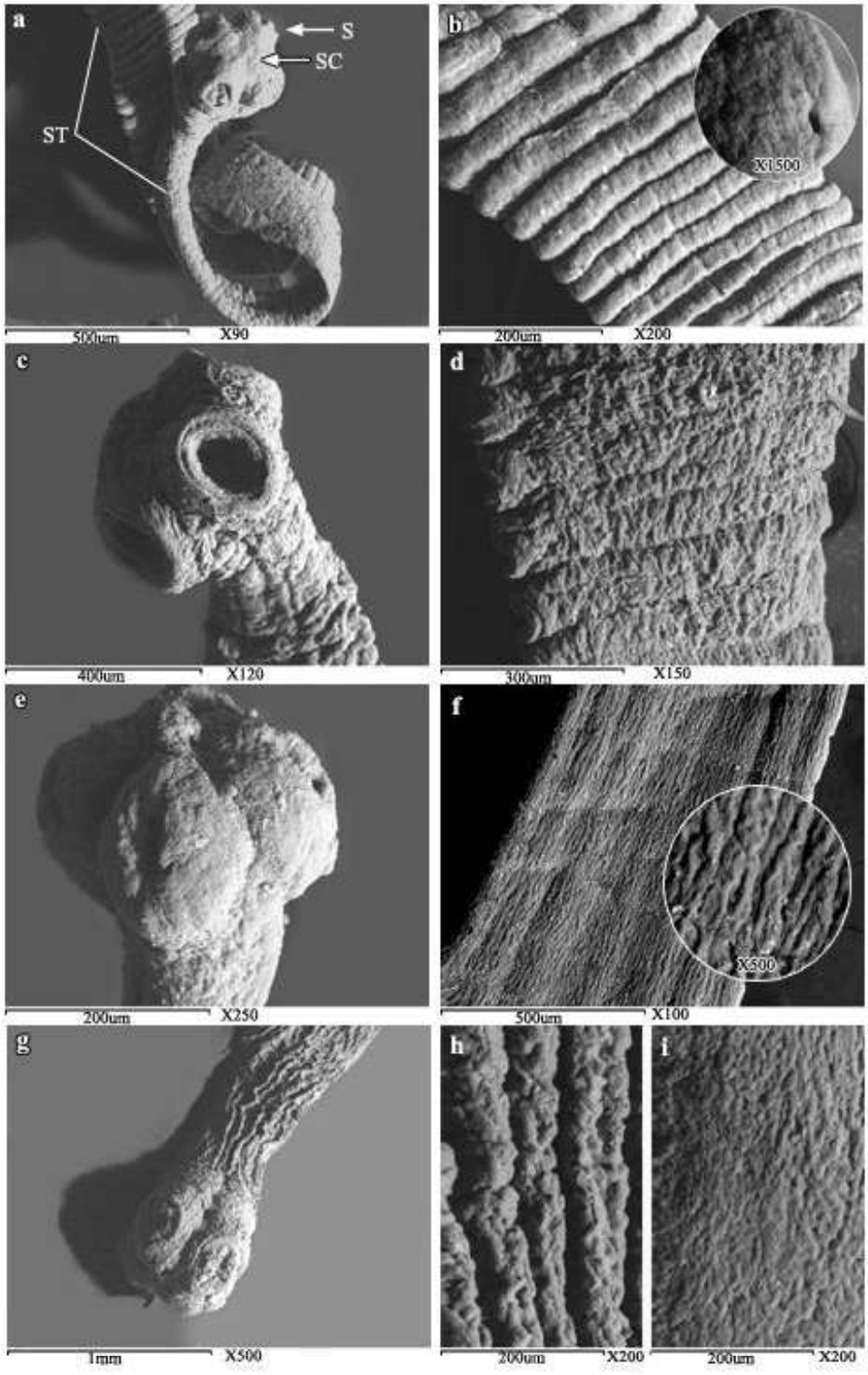

Fig. 2: Scanning electron micrographs (SEMs) of the anterior end of adult *Moniezia expansa*. (a, b) SEMs of normal fresh worm. SC, scolex, S, suckers, ST, strobila (c, d) Changes after 24 h of incubation in 50 ng/ml ivermectin. (e, f) Changes after 24 h of incubation in 1 mg/ml *N. sativa* oil. (g, h, i) Changes after 24 h of incubation in a combination of half-strength ivermectin and half-strength *N. sativa* oil.

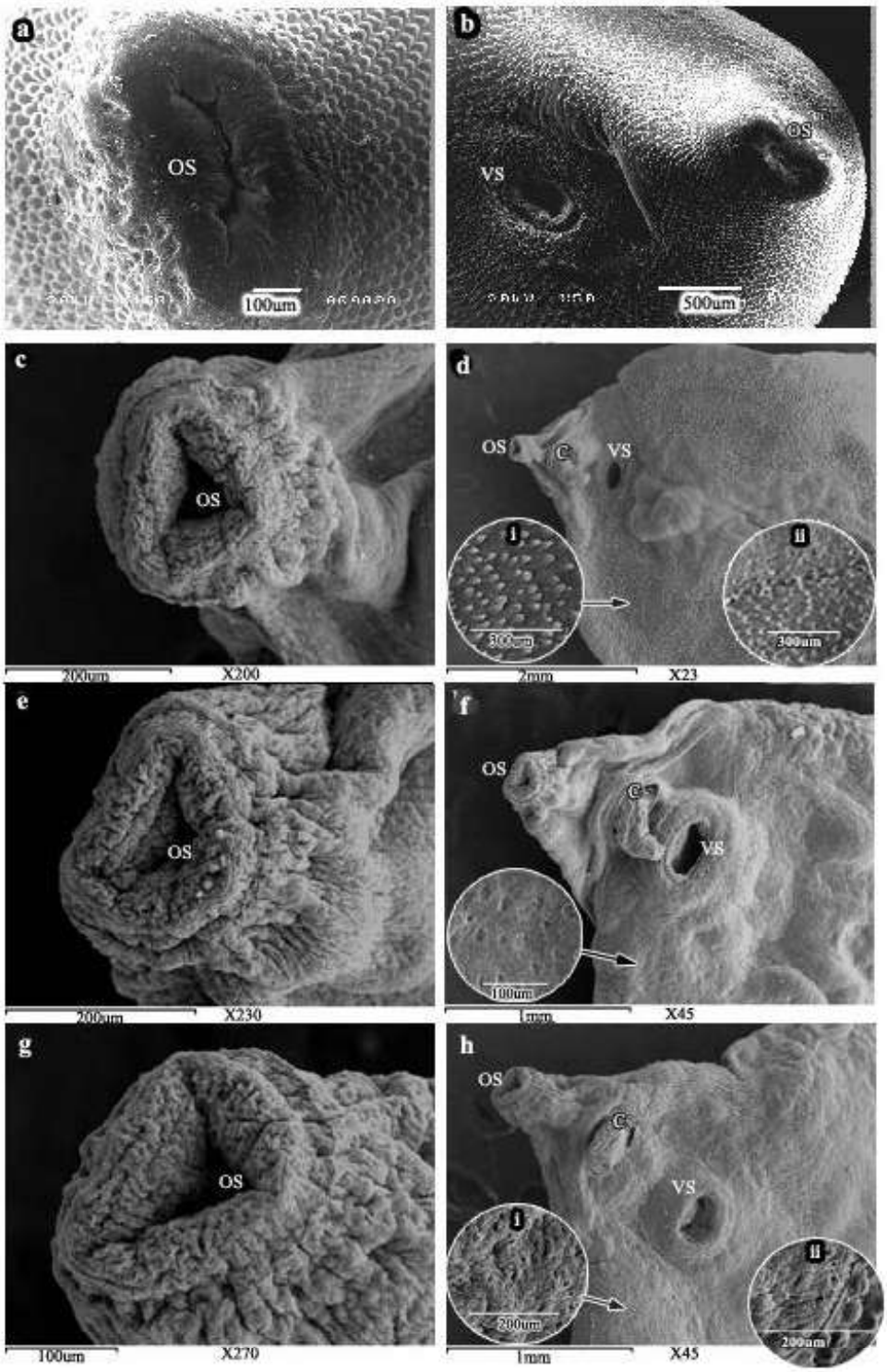

Fig. 3: Scanning electron micrographs (SEMs) of the anterior end of adult *Fasciola gigantica*. (a, b) SEMs of normal fresh fluke revealing smooth oral (OS) and ventral (VS) suckers. (c, d) Changes after 24 h of incubation in 50 ng/ml ivermectin. (e, f) Changes after 24 h of incubation in 1 mg/ml *N. sativa* oil. (g, h) Changes after 24 h of incubation in a combination of half-strength ivermectin and half-strength *N. sativa* oil. OS, oral sucker; VS, ventral sucker; C, cirrus.

After 24 h incubation with 1 mg/ml *N. sativa* oil, the scolex appeared to be more swollen than normal with blebs of varying sizes covering the surface with contractions to the sucker's opening (Fig. 2e). The proglottides appeared deformed with wrinkled surface (Fig. 2f and inset).

The changes in adult cestodes after 24 h incubation with drug combination (25 ng/ml ivermectin + 0.5 mg/ml *N. sativa* oil) concerned the whole-body surface. While the surface of the suckers appeared slightly swollen (Fig. 2g), the general tegument appeared distorted. There was a difference in the degree of disruption in tegumental surface between the examined specimens. In some specimens, severely folded and corrugated tegument was observed (Fig. 2h). In other specimens, tegumental swelling occurred so that the proglottides could not be distinguished (Fig. 2i).

In vitro* Effects of Ivermectin And/or *N. sativa* Oil on Adult *F. gigantica

SEM of normal fresh flukes: The apical cone surface of the normal fresh fluke revealed smooth oral and ventral suckers with thick rims covered with transverse folds. The tegument appeared rough, where at higher magnification; it was ridged with numerous broad and flat serrated spines projecting beyond the surface. The typical intact tegument surface of mid-body region of adult fluke exhibited microridges and grooves with numerous serrated spines. The spines were most numerous anteriorly and decreased in numbers posteriorly (Fig. 3a and b).

***SEM of Treated Flukes*:** After 24 h incubation with 50 ng/ml ivermectin, several specimens showed minor disruption of the tegument. In the oral cone region, the disruption was relatively mild (Fig. 3c). Damage was particularly pronounced in the anterior mid-body region at which swollen tegument was observed on its mid-line part. This was more severe on the ventral surface and areas of tegumental sloughing that were centered on the empty spine sockets (Fig. 3d). The boundaries of these areas were quite sharp the tegumental syncytium remained from which it was removed down to the basal lamina (Fig. 3d inset ii). No disruption was seen toward the lateral margins (Fig. 3d inset i).

After 24 h incubation with 1 mg/ml *N. sativa* oil, there was severe swelling and furrowing of the tegumental surface of the oral cone region (Fig. 3e). As a result of the swelling, the spines appeared sunken and the surface had a wrinkled, corrugated appearance. In the mid-body region, the tegumental surface appeared swollen and severe disruptions were observed at the anterior and the lateral margins of the middle region; at which most tegumental surface was eroded and became spineless (Fig. 3f). This disruption was similar on the ventral and dorsal surfaces.

After 24 h incubation with drug combination (25 ng/ml ivermectin + 0.5 mg/ml *N. sativa* oil), the disruption to the oral cone was similar to that was induced by *N. sativa* oil in most of the examined specimens. In this region, there was widespread swelling of the tegumental syncytium, with many deep furrows (Fig. 3g). In the mid-body region, there was a very distinct boundary, or interface, marking the separation between the anterior mid-body, where the tegumental syncytium had sloughed away to reveal the basal lamina and the posterior mid-body, where the tegumental syncytium remained. Anterior to the interface, the spines were wholly removed from the tegumental syncytium, leaving behind empty spine sockets. Posterior to the interface, the tegumental syncytium appeared swollen and the spines appeared to be partially submerged (Fig. 3h and inset ii). The anterior mid-body region and the lateral margins of the fluke were the most severely and consistently disrupted regions (Fig. 3h inset i). This disruption was similar on both ventral and dorsal surfaces.

Collectively, the cuticular or tegumental disruptions of the worms induced by combination of ivermectin with *N. sativa* oil, in half doses, were more severe than that of ivermectin alone. In all experiments, no differences were observed between fresh and control specimens incubated for 24 h in solvent; 0.03% (v/v) DMSO.

DISCUSSION

The present study demonstrated the comparative morphological effects of ivermectin/*N. sativa* oil combination and each drug separately against helminth parasites; nematode, cestode and trematode representatives. The SEM observations showed that the combination of half dose of ivermectin/*N. sativa* oil was more destructive to the cuticular or tegumental surfaces of adult helminthes than either drug separately. Comparing the ivermectin/*N. sativa* oil -treated *H. contortus* worms with those treated with ivermectin alone, the former were more severely affected with distortion of both buccal capsule and cuticle against distortion of buccal capsule alone in the latter group. Also, the surface changes observed in *M. expansa* and *F. gigantica* after treatment with the drug-combination were more severe than those after treatment with

ivermectin alone, in that tegumental distortion was more widespread, extending into the scolex of the cestode and the lateral margins of the fluke. On the other hand, the surface changes in *H. contortus* induced by *N. sativa* oil were less severe than those described for drug-combination. While, the general level of tegumental disruption in *M. expansa* and *F. gigantica* was similar in both *N. sativa* oil and drug combination-treated groups.

Indeed, ivermectin had a potent antinematodal activity in a variety of domestic animals, but it considered to be insusceptible versus platyhelminths [11]. The antihelminthic activities of *N. sativa* oil were studied by Agarwal *et al*. [15] who reported that the essential oil from the seeds of *N. sativa* showed pronounced activity even in 1:100 dilutions against tapeworms and earthworms. Anticestodal effects of *N. sativa* seeds were studied in children infected naturally with the respective worms. A single oral administration of 40 mg/kg of *N. sativa* ethanolic extract reduced the percentage of the fecal eggs without producing any adverse side effects [16]. When given orally to *Schistosoma mansoni*-infected mice, a 2-week treatment with *N. sativa* oil reduced the number of *S. mansoni* worms in the liver and decreased the total number of ova deposited in both the liver and the intestine [21]. Furthermore, it increased the number of dead ova in the intestinal wall and reduced the granuloma diameters markedly. When *N. sativa* oil was administered in combination with praziquantel, the drug of choice for the treatment of schistosomiasis, the most prominent effect was a further lowering of the dead ova number over that produced by praziquantel alone [21].

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 [22] would probably be responsible for destructive changes and deformation of the nematode body surface [23, 24]. Also, the general body surface of platyhelminth parasites acts as an absorptive surface. In the present study, the helminth's body surface was observed to be affected and altered by *N. sativa* oil and drug-combination. 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 [25-30]. In general, the cuticle of nematodes and the teguments of cestodes and trematodes were known to be the basic entry route and primary site of activity of anthelmintic drugs [22, 31].

N. sativa seeds contained fixed oils and volatile oils, which were rich sources of quinones, unsaturated fatty acids, amino acids and proteins in addition to traces of alkaloids and terpenoids. Most of the studies on the biological effects of *N. sativa* had dealt with its crude extracts in different solvents; however, some studies used its active principles. Among the components isolated from the volatile oil of *N. sativa*, thymoquinone had been shown to be the principal active ingredient [32]. *In vitro* physicochemical assays characterized that component as cytotoxic. This active principle in *N. sativa* oil had been found to exert cytotoxic effects both *in vitro* and *in vivo* against various tumor cells [33]. Therefore, it was reasonable to suggest that the greater disruption to the surface morphology of the combination-treated parasites might be attributed to an additive or synergistic effect between ivermectin and *N. sativa* oil with different modes of action. Few SEM studies had been carried out to determine the synergistic potential of drug combinations. In *F. gigantica* study, Diab *et al*. [34] reported that artemether alone in full dose or combined with ivermectin in half doses had potent fasciocidal activities. Half doses of combined drug regimens had higher ovicidal effect than each drug alone. In two previous studies involving *Echinococcus granulosus*, greater surface disruption was demonstrated with combinations of praziquantel plus albendazole and ivermectin plus albendazole than with the individual drugs [35, 36]. Greater severity of morphological changes induced by the particular combination of drugs compared with either drug by its own, as proved by transmission electron microscope studies, had been interpreted as synergism [37].

In conclusion, the present study had provided morphological evidence for the greater anthelmintic activity of ivermectin on combination with *N.* sativa oil and the results lent support to the idea of using drug combinations against helminthes infections.

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