

## Bioassay of Egyptian Propolis on *Toxocara vitulorum* Adult Worms

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**Abstract:** Indiscriminate uses of chemical anthelmintics have resulted in the establishment of resistant parasites. Thus, this study aimed to evaluate the antiparasitic effect of Egyptian propolis ethanolic extract -as an alternative used drug- on adult *Toxocara vitulorum* *in vitro*. Adult worms were incubated for 24 h. in different concentrations of the extract (100, 50, 25, 12 and 6 mg/ml). The extract revealed anthelmintic efficacy and the mortality rate was concentration dependant. LC<sub>25</sub>, LC<sub>50</sub> and LC<sub>90</sub> were 6.9, 12.5 and 53.4 mg/ml, respectively. The mode of action of propolis ethanolic extract on adult worms was assessed by light and scanning electron microscopy following 24 h. incubation. The disintegration of hypodermis, disorganization of muscle layers and retraction of lips, cuticle damage and distortion of excretory pore were detected and confirmed the nematocidal effect of Egyptian propolis. The overall findings of the current study confirmed effective and natural anthelmintic alternative to the more expensive drugs could be developed using Egyptian propolis.

**Key words:** Egyptian Propolis • *Toxocara vitulorum* • Light Microscopy • Scanning Electron Microscopy

### INTRODUCTION

Widely distributed around the world, *Toxocara vitulorum* (*T. vitulorum*) is a nematode parasite of the small intestine of ruminants, particularly buffalo calves between one and three months of age, causing high morbidity and mortality [1]. Also, this parasite has zoonotic importance, where humans become infected by ingestion of infective eggs either from soil, dirty hands, raw fruits and vegetables or larvae from unpasteurized milk [2]. Larval migration through different soft tissues in the human causes several clinical diseases in the patient, such as visceral larva migrans, ocular toxocarosis and neurotoxocarosis [3]. The therapy of toxocariasis is based largely on chemical drugs [4]. However, these drugs generate severe side effects, including resistant parasites, chemical residues in host tissues and environmental pollution. Such problems diverted the researcher attentions towards the development of alternate methods for the treatment of helminthiasis [5]. So, the discovery of new compounds with nematocidal activity and immunomodulatory properties is essential for the

development of new safe alternative drugs to toxocariasis therapy. Many previous trials were concerned with the treatment of *T. vitulorum* by natural products. One from these products is propolis, a resinous substance that bees collect from the exudates of plants and which they use to seal holes in the bee-hive [6, 7]. Propolis also forms part of traditional medicine and chemical analysis has pointed to the presence of at least 300 compounds in its composition [8]. It is mainly composed of resin (50%), wax (30%), essential oils (10%), pollen (5%) and other organic compounds (5%) [9]. Among these organic compounds are phenolic compounds, esters, flavonoids in all their forms, terpenes, beta-steroids, aromatic aldehydes, alcohols, sesquiterpenes and stilbene terpenes [10]. Propolis composition varies with different factors, such as a source of the exudates, climate and environmental conditions [11, 12]. Caffeic acid phenethyl ester (CAPE) is a biologically active ingredient of propolis with several interesting biological properties, including apoptosis [13] metastasis [14] antiviral [15, 16] and antimicrobial activities [17, 18]. In addition, propolis proved anti protozoal effect where Brazilian propolis can reduce

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*Leishmania amazonensis* infection in macrophages cultures [19]. Moreover, in our laboratory it exhibited anthelmintic activity against *Fasciola gigantica* adult worm, it causes distortion of oral and ventral suckers, tegumental lesion and loss of spines [20] and proved inhibitory effect on the vitality and hatchability of immature *F.gigantica* eggs [21]. Also, it could increase the level of protection against infection with *Taenia saginata* in mice when administered at the same time with immunization [22]. So, the principle goal of the present study is to evaluate the *in vitro* effect of propolis on *T. vitulorum* adult worms and the changes were assessed by both light and scanning electron microscopy.

## MATERIALS AND METHODS

**Propolis:** Propolis sample was collected from beehives located in Giza Governorate, Egypt. The sample was kept in the dark and stored at -20°C until used.

**Preparation of Propolis Ethanolic Extract:** Fifty grams of the resinous material of Egyptian propolis was cut into small pieces and extracted with 250 ml of 80% ethanol. The extraction process was carried out according to the method of Hegazi *et al.* [23]. The alcoholic extract was evaporated under vacuum at 50 °C until dryness. Dried ethanolic extract (5 g yield) of propolis was suspended in PBS (pH 7.2).

**Recovery of Adult Worms:** Adult worms of *T. vitulorum* were collected from the small intestine of naturally infected buffalo calves slaughtered in Cairo abattoir. After recovery, adult worms were washed several times with distilled water and transfer to phosphate buffered saline (pH 7.2) to remove any adherent fecal materials.

**Assessment of Propolis Nematicidal Activity *In vitro*:** *T. vitulorum* adult worms were cultured in PBS with 0.1% Dimethyl Sulfoxide (DMSO) containing antibiotics (Penicillin, 100 IU/ml; Streptomycin, 100 µg/ml) (Sigma - Aldrich Chemie GmbH, Germany) and propolis ethanolic extract at different concentrations (100, 50, 25, 12 and 6 mg/ml). DMSO and normal control worms were maintained in the same medium without propolis extract and under the same laboratory conditions. Treated and control worms were maintained at 37 °C and 5 % carbon dioxide in a CO<sub>2</sub> incubator. Five replicates of each concentration and five worms for each replicate were used. After incubation for 24 h LC<sub>25</sub>, LC<sub>50</sub> and LC<sub>90</sub> were calculated.

**Light Microscopy:** After incubation, the adult worms (treated and control) were cut into small, 5mm, pieces before fixed in 10% formal saline. After dehydration, samples were embedded in paraffin and sectioned at 4-6 µ. Sections were stained with hematoxylin and eosin [24]. The structure of body wall of adult worms was studied and photographed using an Olympus CX41 microscope.

**Scanning Electron Microscopy:** Adult worms of *T. vitulorum* (treated and control) were washed several times with double distilled water. The specimens were fixed for 12 h in a 3:1 mixture of 4% (w/v) glutaraldehyde in 0.12M Millonig's buffer, pH 7.4 and 1% aqueous osmium tetroxide. Dehydrated by acetone, critical point dried in carbon dioxide then fixed to aluminum stubs and coated with gold-palladium. The specimens were viewed in a jeol scanning electron microscopy operated at 15 kV according to the methods of Shehab *et al.* [25].

## RESULTS

**Naked Eyes Observations:** During the time of the experiment, untreated *T. vitulorum* showed full motility by naked eyes. Thus, after 24 h of incubation, they showed the same fitness as at the beginning of the incubation.

**Effect of Propolis Extract on *T.vitulorum* Adult Worms:** Propolis ethanolic extract exhibited anthelmintic activity against *T. vitulorum* adult worms and the mortality rate was concentration dependent (Fig. 1). LC<sub>25</sub>, LC<sub>50</sub> and LC<sub>90</sub> were 6.9, 12.5 and 53.4 mg/ml, respectively.

### Light Microscopic Observations

**Control Worms:** *T. vitulorum* adult worms, which had been incubated in PBS without propolis or DMSO, did not show any damaged structures, even after 24 h incubation (Fig 2a). This result was also observed in worms that had been incubated in PBS containing 0.1% DMSO (solvent treated control worms).

Histology of the body wall of normal *T. vitulorum* revealed that the cuticle was composed of several proteinaceous layers running continuously around the body. Below the cuticle is a syncytial epidermis (Hypodermis), followed by a complex muscular layer. The muscular layer consistent from two distinct portions: a fibril contractile muscular portion directed toward hypodermis and a granular non-contractile protoplasmic portion projecting toward the center of the body. Some microfibrils were observed in contractile part of muscle cells (Fig. 2a).

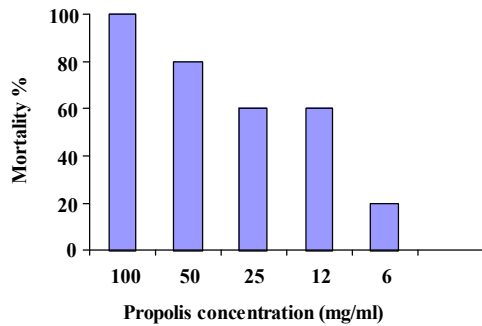


Fig. 1: Mortality rate of *Toxocara vitulorum* treated with different rate of Egyptian propolis ethanolic extract at 24 h post treatment

**Treated Worms:** Propolis (100 mg/ml) treated *T. vitulorum* worms exhibited disorganization of cuticle structure. The disappearance of microfibrils in the contractile part of muscle cells and damage of non contractile part were detected (Fig 2b). Swollen and severe vacuolization of hypodermis was observed, this vacuolization reached to complete disappearance of hypodermis structure (Fig. 2c). Consequently, there was a disconnection between the outer cuticle and inner damaged muscular layer (Fig. 2d).

### Scanning Electron Microscopic Observations

**Control Worms:** Scanning electron micrographs showed the characteristic features of adult *T. vitulorum*. There were three prominent lips having smooth cuticle and appeared anchored to one another, one dorsal and two sub ventral, surrounding the mouth opening (Fig. 3a). The inner surface of each lip has a single dentigerous ridge which was composed of a single line of minute unicuspid denticles (Fig. 3b). The body wall of adult worm showed a smooth cuticle characterized by fine corrugation giving rise to a series of characteristic transverse striations called annulations. Those form a continuous ring around the body called annuli, which occurred at regular intervals giving the body a segmented appearance. These annuli separated by interrupted smaller grooves called sub annuli (Furrows) (Fig. 3c). Excretory pore situated on the ventral surface at the first third of the body (Fig. 3d). The cuticle can be sub-divided into the broad dorsal and ventral regions covering the dorsal and ventral hypodermis which connected to each other by seam cells where longitudinal ridges termed alae are found on the cuticle. Abnormal changes were not observed in control worms.

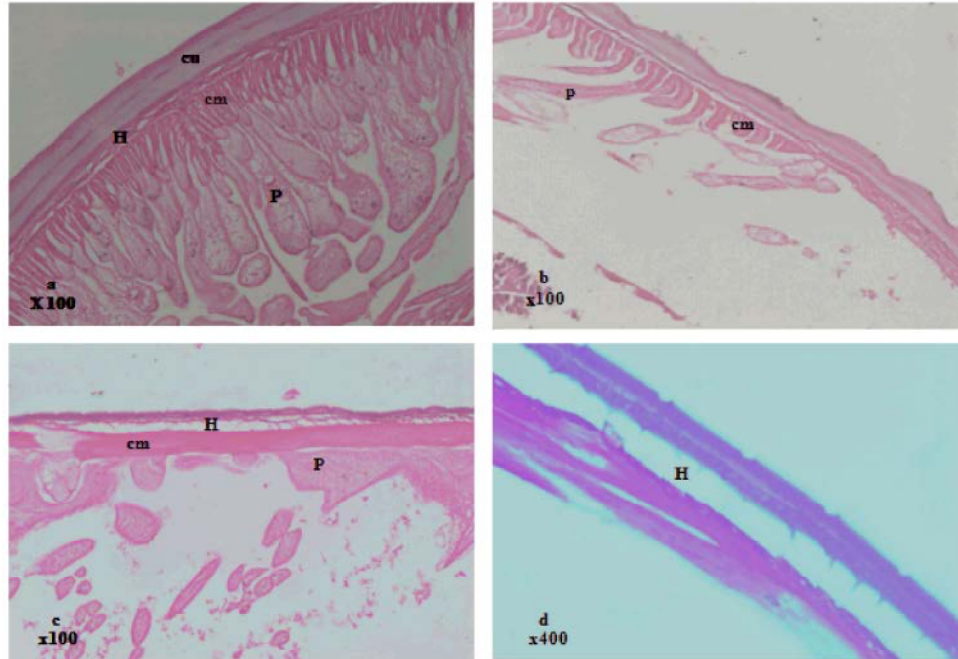


Fig 2: Light micrograph sections of *Toxocara vitulorum* adult worms (Control and treated). a: Cross section in normal control worm. b: Cross section in treated worm showed damage of contractile and noncontractile muscle layers. c: Longitudinal section in treated worm showed sever vacuolization of hypodermis and distortion of muscle layers. d: Longitudinal section in treated worm showed the disappearance of hypodermis and disconnection between the outer cuticle and inner destroyed muscle layers. cu, cuticle; H, hypodermis; cm, contractile muscle; p, protoplasmic portion

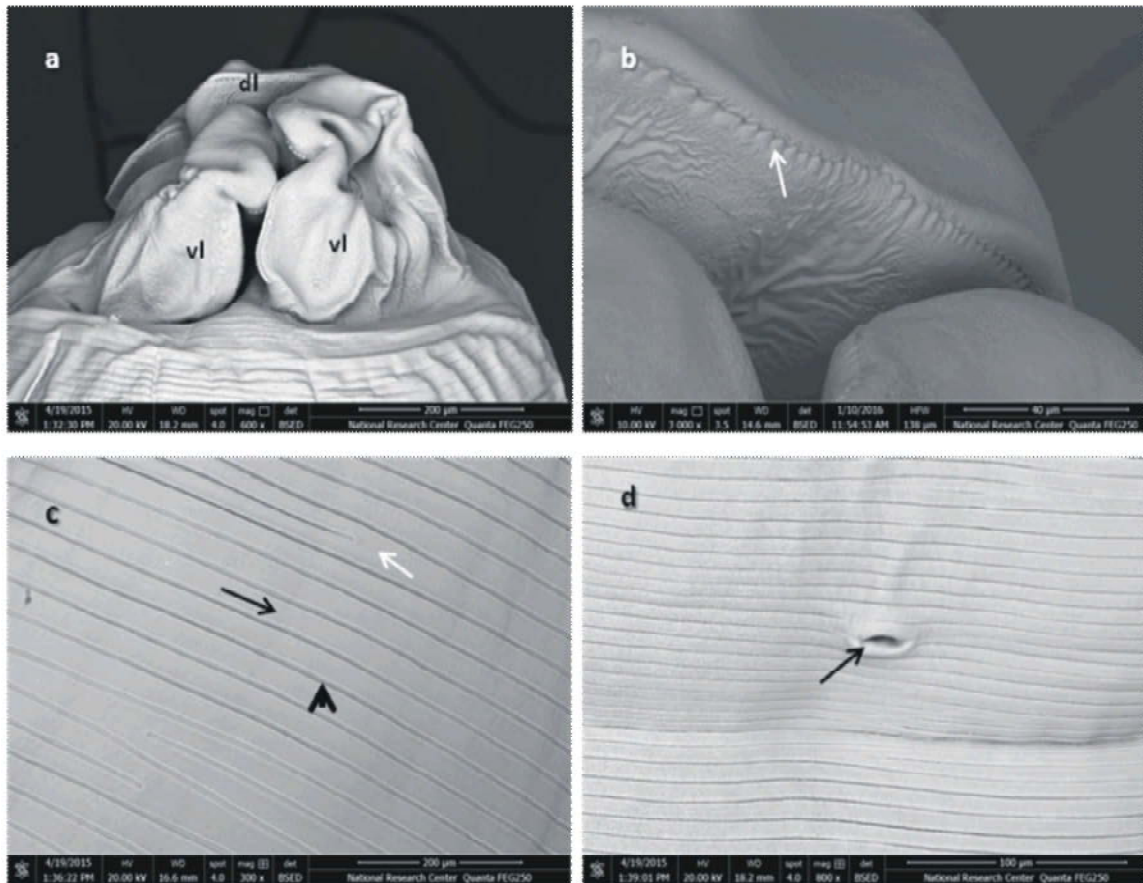


Fig. 3: Scanning electron micrographs (SEMs) of control adult *Toxocara vitulorum* body surface: a: Anterior end of normal control worm revealed three prominent lips, one dorsal (dl) and two sub ventral (vl). b: Higher magnification of dorsal lip showed minute unicuspid denticles on its inner surface (White arrow). c: SEM of normal cuticle showed annuli (Black arrow), sub annuli (Black arrow head) and branched annuli (White arrow).d: SEM of normal worm showed excretory pore (Black arrow)

**Treated Worms:** After 24 h exposure to propolis extract(100 mg/ml) *in vitro*, worms revealed swelling, retraction of lips, an appearance of internal fibril structure of oral cavity and distorted sensory papillae. Wrinkling appearance in addition to some lesions in cuticle surface of lips and removing of some lip denticles were observed (Figs. 4a & b). Corrugated cuticular surface and the annuli were strongly separated from each other compared with untreated worms (Fig. 4c), lesions, adhering and cracks of cuticle were observed (Fig. 4d). Also, protrusion and deformity of excretory pore were detected (Fig. 4d).

## DISCUSSION

The use of natural products for safety curative purposes has a long history and compounds which isolated from natural products actually played an important role in the pharmaceutical industry [26]. In

addition to microbes and plants, there has been growing interest in other natural products such as propolis which considered as an important source of biologically active compounds [17]. However, the potential use of this natural product as a source of the new anthelmintic drug is still poorly explored. So, in the present study, we have evaluated the nematicidal effect of Egyptian propolis on *T. vitulorum* adult worms and assessed its effect by light and scanning electron microscopy. Initially, there was no difference in cuticle structure of solvent treated and normal control worms. This was coincided with that previously reported by Mehlhorn [27] and Shalaby *et al.* [28]. Therefore, the damage or structural changes observed on the worms after propolis extract application were only due to the effect of this extract. After 24 h incubation with propolis, disorganization of cuticle and damage of muscular layers were detected by light microscopy. Also, scanning electron microscopy

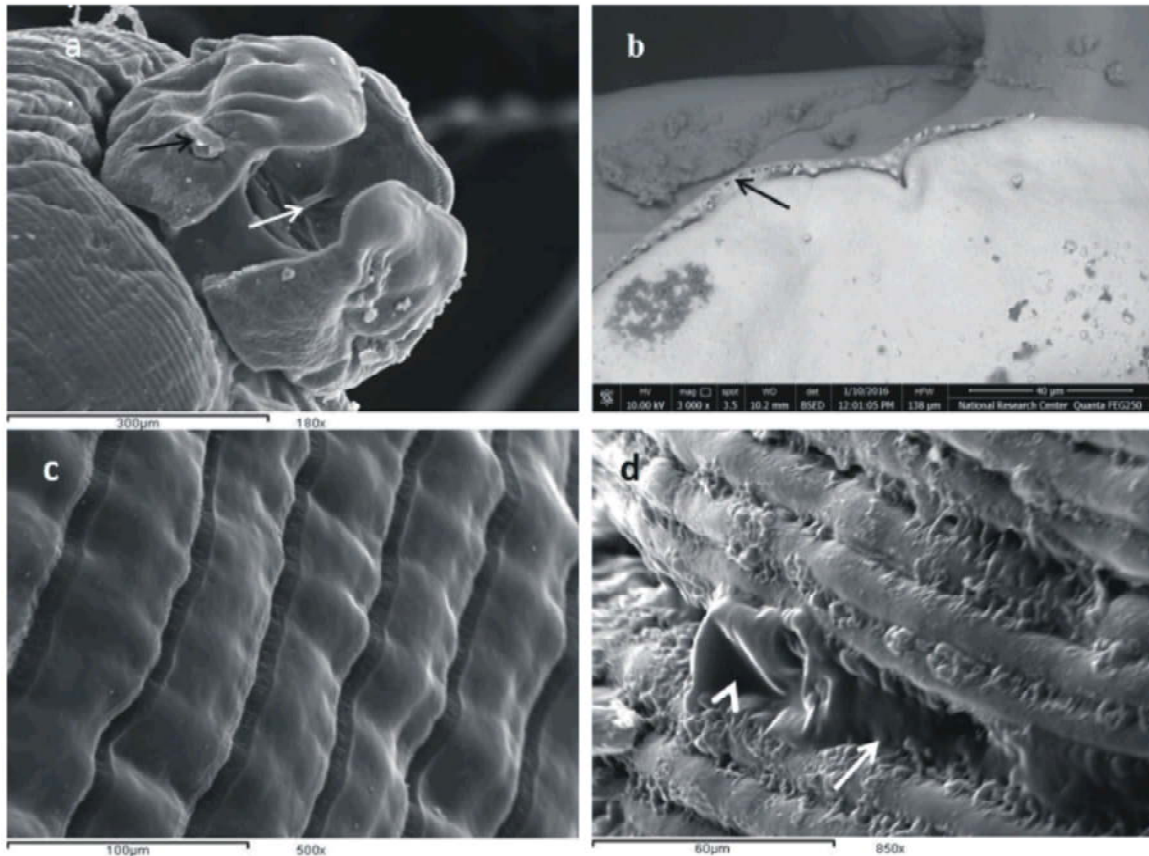


Fig. 4: Scanning electron micrographs (SEMs) of treated adult *Toxocara vitulorum* a: Anterior end of treated worm showed retraction of lips, appearance of internal fibril structure (White arrow) and distortion of sensory papillae (Black arrow). b: Higher magnification showed removing of some minute denticles of worm lips (Black arrow). c: SEM of treated worm showed wrinkled cuticle surface. d: SEM of treated worm showed cracks of cuticle (White arrow) and distortion of excretory pore (White arrow head)

confirmed nematicidal effect of propolis, where retraction of lips and its wrinkled cuticle surface, deformed of sensory papillae, lesions, adhering and cracks of cuticle in addition to protrusion and deformity of excretory pore were detected. This destructive effect was similar to that observed on the tegumental structure of *Fasciola gigantica* treated with Egyptian propolis [20]. Egyptian propolis not only affected adult stage of *Fasciola gigantica* but also proved evidenced inhibitory activity on the vitality and hatchability of its immature eggs [21]. Also, disorganization of cuticle and body musculature was observed in *T. vitulorum* treated with *Balanites aegyptiaca* fruits [29]. Moreover, *in vitro* studies on another nematode, *Ascaris suum*, showed similar cuticular damage after exposure to the alcoholic extract of *Lysimachia ramosa* [30]. In addition, similar destructive alterations and deformity in the cuticle of *Haemonchus contortus* and also in the tegumental architecture of

*Moniezia expansa* treated with *Allium sativum* oil were detected [31]. Destructive tegument was also observed in *Fasciola gigantica* treated with *Meryta denhamii* stem ethanolic extract [25]. The similarity of damage in the presently treated worms with that of the same or other parasites treated with plant extracts might be due to the botanical origin of propolis. Moreover, in the present study the change of structure architecture of treated *T. vitulorum* was in harmony with that reported in nematode species treated with chemical drugs *in vitro* by Shalaby *et al.* [29] and An [32]. Based on a theory that anthelmintics entered to target parasites by oral ingestion or by diffusion through the external surface [33] passive diffusion of anthelmintics through the cuticle or tegument might be responsible for destructive changes and deformation of the nematodes, trematodes and cestodes body surface [34]. The cuticle in nematodes or tegument in trematodes and cestodes is metabolically active and



morphologically specialized for selective absorption of nutrients, secretion of glycoprotein for protection, osmoregulation and sensory perception [35]. In the present study, treatment of worms with propolis might disrupt the previously mentioned physiological processes as a result of cuticle irreversible damage which might facilitate penetration of propolis extract to deeper tissues causing greater and wide spread damage which led to worms death. Collectively, the overall findings of the current study indicated that Egyptian propolis has high effectiveness against adult stage of *T. vitulorum*. This encourages further *in vitro* and *in vivo* investigations to determine optimal strategies for use this natural product for the treatment of toxocariasis.

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