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Curcumin Impact on Ex Vivo *Toxocara vitulorum* Adult Worms and Eggs

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ABSTRACT
Conventional drug treatment expels adult worms from the intestinal tract but does not prevent reinfection and further contamination of the environment with eggs. This study was performed as the start of a program aiming to develop curcumin (CUR) as a lead drug for human and animal ascariasis. Ex vivo adult *Toxocara vitulorum* and eggs were exposed at 37°C for 4hr to different concentrations of CUR (0-500µM) in Roswell Memorial Park Institute (RPMI) 1640 medium supplemented with 2% dimethyl sulfoxide (DMSO). The results showed that CUR slightly impaired the viability and mobility of approximately 30% of worms in a dose-dependent manner. Scanning electron microscopy revealed changes in cuticles throughout the length of the roundworms exposed to high CUR concentrations. Enzyme-linked immunosorbent assay failed to reveal CUR effects on specific antibody binding to the roundworm surface membrane molecules. To mimic the in vivo situation, after incubation, eggs were washed, suspended in 0.1N sulphuric acids, and examined for development and maturation 4, 7, 11, and 18 days later. Eggs produced from CUR-exposed females showed an even more remarkable delay and impairment in development in comparison to eggs, which were directly exposed to CUR. A significant increase in the number of dead eggs and prevention of maturation to the infective stage were observed in a CUR dose and time-dependent manner. These results encourage investigations of CUR impact on human and animal ascarids of public health and economic importance.

Key words: Ascarids, Curcumin, Eggs development, Ex vivo worms, Scanning electron microscopy, *Toxocara vitulorum.*

INTRODUCTION

Parasitic nematodes of the family Ascarididae affect humans and various mammals including pigs and ruminants. *Ascaris lumbricoides* is the most prevalent nematode infection of man where it globally infects from 800 to 1.2 billion people (approximately 15% of the world's population), with children (320 million) being the most susceptible. Infection results in impaired nutrition, reduced protein, fat, vitamin A, and iodine absorption, anorexia, intestinal pathological changes and obstruction (Dold and Holland 2011; Wright et al. 2018).

Ascaris lumbricoides is derived directly from *Ascaris suum*, which usually infects pigs (Leles et al. 2012; da Silva Alves et al. 2016; Nagorny et al. 2019). Cases of *A. suum* infections were recorded in cattle and water buffaloes, the reservoirs of the ascarid, *Toxocara vitulorum* that also infects man (Abdel-Rahman and El-Menyawe 2015; Taylor et al. 2016; Ma et al. 2018). Ruminant calves toxocariasis is initiated via trans-placental and trans-mammary routes and characterized by loss of appetite, weight loss, diarrhea that leads to morbidity and high mortality rate, and results into serious economic losses (Rast et al. 2013). *Toxocara vitulorum* also infects man, likely via cow milk, leading to serious clinical entities, such as visceral and ocular larval migrans, and neurotoxicariasis (Abdel-Rahman and El-Menyawe 2015; Ma et al. 2018; Meliou et al. 2020; Raissi et al. 2020, 2021).

Current anthelmintics are used to manage ascariasis and associated complications. In humans, a single dose of albendazole or mobendazole twice daily for 3 days is the usual anti-helminth therapy (Hagel and Giusti 2010). Additionally, the ovocidal activity of benzimidazole thiabendazole (Egerton 1961), benzimidazole albendazole (Boes et al. 1998) and flubendazole (Zhao et al. 2017) against eggs of *A. suum* was elucidated in pigs. However, reduced efficacy, serious side effects, and drug resistance (Wolstenholme et al. 2004; Abdel-Rahman and El-Menyawe 2015; Ma et al. 2018) led to the search for alternative ascaricides from natural sources, mainly plant-derived compounds (Hoste et al. 2006; Shalaby et al. 2012; Bazh and El-Bahy 2013; Shalaby and El-Moghazy 2013).

Medicinal plants, especially turmeric, are safe, low cost, naturally occurring phytochemical product widely used in India and China. Turmeric is obtained from the rhizomes of the Zingiberaceae, *Curcuma longa* L

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(Bhowmik et al. 2009). Traditional turmeric uses in India include improving digestion and intestinal flora and eliminating intestinal worms (Prasad and Aggarwal 2011; Kumar and Sakhya 2013; Sobhy et al. 2021). The active ingredient in turmeric is curcumin (CUR) (diferuloylmethane), which is commonly used as a spice and in the food industry (Singh 2007; Kumar and Sakhya 2013; Tsuda 2018). CUR has been proposed to prevent and treat inflammatory, rheumatic, neurodegenerative, ocular, neoplastic, viral, bacterial and parasitic diseases (Zhou et al. 2011; Kumar and Sakhya 2013; Pescosolido et al. 2014; Tsuda 2018; Xu et al. 2018; Abou El Dahab et al. 2019). The promise of this substance in alleviating numerous afflictions and promoting human health has been bitterly challenged by Baker (2017), who has nevertheless reported that CUR has the capacity of disrupting cell membranes, a required mechanism for tumoricidal and microbicidal action, provided specificity, safety and lack of toxicity, the paramount CUR features (Kumar and Sakhya 2013; Pescosolido et al. 2014; Nelson et al. 2017a; Xu et al. 2018).

The main objections raised against CUR effectiveness were essentially attributed to its poor solubility and hence limited systemic bioavailability, and to remarkable instability, being readily biotransformed by human intestinal cells and microflora to yield oxidative, glucuronides and sulfate O-conjugated derivatives (Singh 2007; Pescosolido et al. 2014; Baker 2017; Nelson et al. 2017a, b; Xu et al. 2018; Zam 2018). Indeed, after oral administration, CUR shows preferential distribution and accumulation in the intestine (Nelson et al. 2017a; Shen and Ji 2019). Accordingly, CUR is likely active in the gut, as it may directly come into contact with the gut microbes and mucosa without need of systemic absorption. The various metabolites of CUR were also shown to be biologically active and make significant contribution to its diverse beneficial actions (Shen and Ji 2012; Edwards et al. 2017; Tsuda 2018; Chieffi et al. 2021). Importantly, recent reports have revealed that CUR effects might be mediated by, or synergized with, its action on the gut microbiota (Zam 2018; Shen and Ji 2019).

In our efforts to develop CUR as a lead drug for ascariasis, we started by assessing its *in vitro* effects on the *ex vivo* adults and eggs of *T. vitulorum* and revealed its impact on adult worms at the ultrastructural level, and its impairment of the viability and development of eggs derived from CUR-exposed worms or following direct exposure.

MATERIALS AND METHODS

Ethical Approval

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals. Protocols and procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Science, Cairo University, License number CUFS-S-Immu-22-16.

Animals and Parasites

Female C57BL/6 mice (6–8-week-old) were raised at the Schistosome Biological Materials Supply Program, Theodore Bilharz Research Institute (SBSP/TBRI, Giza, Egypt) and kept in the animal facility of the Zoology Department, Faculty of Science, Cairo University, Giza, Egypt, during the experiment. Adult *T. vitulorum* (male and female) were extracted from the intestines of infected calves from a slaughterhouse (El-Basateen, Giza, Egypt), and delivered to the laboratory very immediately (within less than 1hr). Worms were washed in normal saline solution ten times before being placed in RPMI 1640 medium [RPMI 1640 contained 1mM sodium pyruvate, 2mM L-glutamine, 200U/mL penicillin, and 200 µg/ml streptomycin] (Invitrogen, Carlsbad, California).

Curcumin Anthelmintic Impact on Adult Worms *In Vitro*

Worm Viability

Adult *T. vitulorum* were distributed equally (5 worms each). All groups were incubated at 37°C for 4 hr. The first group was incubated in RPMI/10% fetal calf serum (FCS) as control group (C). The second group was incubated in RPMI medium/10% FCS supplemented with 2% dimethyl sulfoxide (DMSO) and considered as solvent control (C-DMSO). The CUR-treated (CUR-T) groups were incubated at 30, 60, 125, 250, and 500 µM CUR in culture medium and 2% DMSO (Sigma-Aldrich, St. Louis, Missouri). Worms were examined by light microscopy. When worms did not move, they were shaken vigorously and/or exposed to light before being considered nonviable (Husori et al. 2016). The experiment was repeated twice.

Scanning Electron Microscopy (SEM)

Controls (C and C-DMSO) and CUR-T (500µM) adult worms were fixed in 3% glutaraldehyde in sodium cacodylate buffer (pH 7.2) for 2hr, post-fixed in 1% osmium tetroxide for 2hr at 4°C (all from Sigma-Aldrich), and dehydrated in an ascending series of ethanol then acetone. Specimens were mounted on aluminum stubs, coated with gold (Jeol JFC-1100 E), then examined with a Jeol JEM-1200 EXII electron microscope (Mitaka Supply Co., Ltd, Osaka, Japan) (Ashour 1994).

Antigenicity of Adult Worms Surface Membrane Molecules

Antigen Preparation

Triton-soluble surface membrane antigen preparations (TSAP) of intact and 500µM CUR-treated worms were prepared as described by Oaks et al. (1981). Worms were incubated in RPMI medium supplemented with 0.2% TritonX-100 (Promega, Madison, Wisconsin), 1 µg/mL leupeptin and 5mM phenyl methyl sulfonyl fluoride (Sigma) as protease inhibitors, at ratio of 1:6 (worm:medium), and were shaken on ice for 25min followed by 1min vortexing. The suspension was centrifuged at 400*g* for 10min, and the supernatant, TSAP, was aliquoted and stored at -20°C until use. The Bradford assay (Bio-Rad, Hercules, California) was used to estimate the protein content.

Immunization and Serum Preparation

Five C57BL/6 mice were intramuscularly immunized 3 times, with 3 week-intervals, with 50µg of TSAP from intact worms (TSAP intact) using aluminum hydroxide gel (13mg/mL, Sigma) as adjuvant in first and second immunization. All mice were bled individually via tail vein, before (0-day, control serum) immunization, and via

brachial vein, 7 days after the last, adjuvant-free immunization. After clotting, the blood was centrifuged at 400*g* for 20min. The supernatant (serum) was stored at -20°C until use.

Indirect Enzyme-Linked Immunosorbent Assay (ELISA)

Indirect ELISA was performed as described (Abou El Dahab et al. 2019). Briefly, ELISA plates (Costar, Cambridge, Massachusetts) were coated in parallel with 1µg/well TSAP from intact and CUR-treated adult *T. vitulorum* in carbonate-bicarbonate coating buffer (pH 9.6), overnight at 4°C. After washing, and 1hr incubation with serum samples (1:1,000 to 1:8,000), the plates were incubated with horseradish peroxidase (HRP)-labeled goat anti-mouse immunoglobulin (Ig)G (H+L) conjugate (1:5,000 dilution, Promega). SureBlue 3, 3`, 5, 5-tetramethyl-benzidine (TMB) Microwell Peroxidase Substrates was used to develop the reaction (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, Maryland) at room temperature for 30min, and the colored developed was measured spectrophotometrically at 650nm.

Egg Viability and Development

Eggs were isolated from uteri of longitudinally dissected 10 adult females *T. vitulorum* as described by Boes et al. (1998). The worms were collected from calves' intestine in an hour, washed and transported to our laboratory in ice cold saline, then washed with RPMI medium. The uteri were cut, opened and fragmented for eggs recovery. The eggs were washed with Dulbecco's phosphate-buffered saline, pH 7.1 (D-PBS), sieved, centrifuged and re-suspended in 0.1N sulphuric acid (H_2SO_4) as antifungal solution, then were investigated with 40x inverted microscope (Olympus, Tokyo, Japan) for integrity and viability by Trypan blue staining (Karkashan et al. 2015).

The eggs were placed in tissue culture flasks (Costar) suspended in 25mL, 0.1N H_2SO_4 at a final concentration of about 2,000eggs/mL. The level of the solution was marked on the flask and deionized water was added to maintain the desired concentration in case evaporation was observed. The flasks were incubated at 28°C in the dark for 18 days. Every day, the incubator's temperature was checked, and the samples were mixed by hand to allow oxygenation to proceed. A total of 100eggs/sample were microscopically (x10 and x40 magnification) investigated daily for studying the developmental stages and establishment of development stages charts (Cruz et al. 2012; Shalaby et al. 2012; Schmitz et al. 2016; Abou-El-Naga 2018).

***In Vitro* Anthelmintic Effect of Curcumin on *T. vitulorum* Eggs**

Indirect Curcumin Effect on Spawned Eggs

For each of 2 separate experiments, adult females *T. vitulorum* were exposed to different CUR concentrations from 0 to 500µM for 4hr at 37°C. All worms were washed then kept in D-PBS for 24hr at 28°C to allow eggs production (spawning). Eggs released were collected, washed with D-PBS and counted. Eggs were equally divided, incubated in 0.1N H_2SO_4, in the dark, at 28°C in a humidified atmosphere for 18 days. A total of 100 eggs/sample were microscopically (x10 and x40 magnification) investigated on day 4, 7, 11 and 18 for studying CUR effects on embryonic developmental stages and viability (Cruz et al. 2012; Shalaby et al. 2012; Schmitz et al. 2016; Abou-El-Naga 2018).

Direct Curcumin Effect on Eggs

Eggs were collected from freshly dissected females, and washed with D-PBS. Aliquots of equal egg numbers were suspended in the presence of pure RPMI medium (C), and RPMI medium/2% DMSO containing 0 (C/DMSO) or 30, 60, 125, 250, and 500µM CUR at 37°C for 4hr. All egg samples were washed twice in D-PBS, and suspended in 0.1N H_2SO_4. Intact and treated eggs were incubated in the dark at 28°C in a humidified atmosphere for 18 days and a total of 100 eggs/sample were microscopically (x10 and x40 magnification) investigated on day 4, 7, 11, and 18 for viability using the trypan blue test, and embryonic development (Cruz et al. 2012; Shalaby et al. 2012; Schmitz et al. 2016; Abou-El-Naga 2018).

Statistical Analysis

All values were tested using Students' *–t-* 2-tailed test to analyze the statistical significance of differences between selected values and considered significant at $P < 0.05$ (InStat, San Diego, California).

RESULTS

Ascaricidal Curcumin Activity

Viability, Mobility and Contractility

Over 2 independent experiments, the effects of CUR on both adult male and female *T. vitulorum* were analyzed with respect to the concentration and exposure time. It was difficult to visually detect the roundworms mobility and contractility before and after exposure to CUR. However, x4 and x10 microscopy revealed worms exposed to 250 and 500µM CUR for 4hr were immobile compared to the control ones. In contrast 30, 60, and 125µM CUR did not show any effect on motility and contractility of the *T. vitulorum* for up to 4hr incubation period (data not shown).

Scanning Electron Microscopy

Fig. 1 shows SEM images for adult female and male worms untreated and treated with CUR. In normal untreated worms (C and C/DMSO), the head end consisted of 3 lips, 2 widely rounded lateral and the largest dorsal one. The anterior margin of the lips is flattened (Fig. 1A). The cuticle was transversally striated with normal cuticular annulations in both female and male adult worms (Fig. 1B). Exposure of *ex vivo* adult *T. vitulorum* to 500µM CUR for 4hr caused slight ultrastructural changes in mouth parts and cuticle. The changes in mouth parts involved mainly the lips, which appeared enlarged and dilated in female worms (Fig. 1C, female), and compressed and wrinkled in male ones (Fig. 1C, male). On the other hand, the effect of CUR on the female cuticular structure was seen as loss of some cuticular striations (25±4.95µm) due to swelling and oedema, accompanied by increase in the interannular spaces (Fig. 1D, female). Wrinkled cuticle was seen throughout the length of the CUR-treated male worms (Fig. 1D, male)

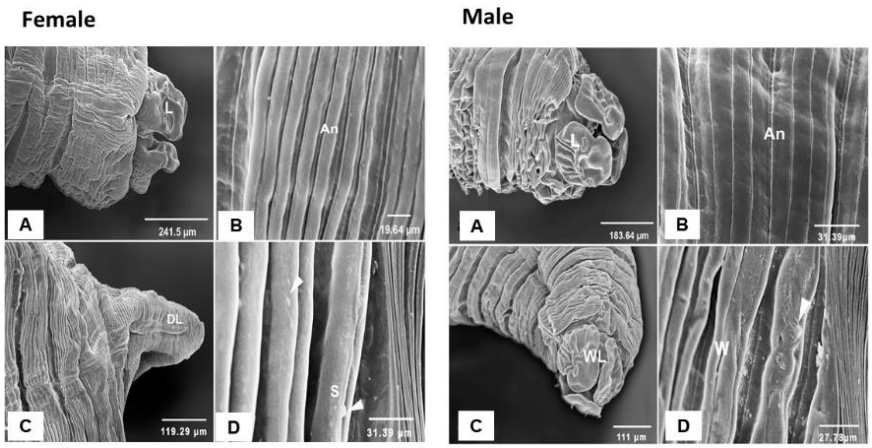

Fig. 1: Electron-scanning microscope imaging of adult *Toxocara vitulorum* worms. The figure shows the outer membrane of adult female and male *ex vivo* worms following incubation in RPMI medium supplemented with (A) 10% FCS alone or with (B) 2% DMSO and (C&D) 500µM curcumin for 4 hr. L=mouth parts-lips; DL=dilated lips; WL=wrinkled lips; Arrowhead, lesion; An=cuticular annulations; S=swelling; W=wrinkling.

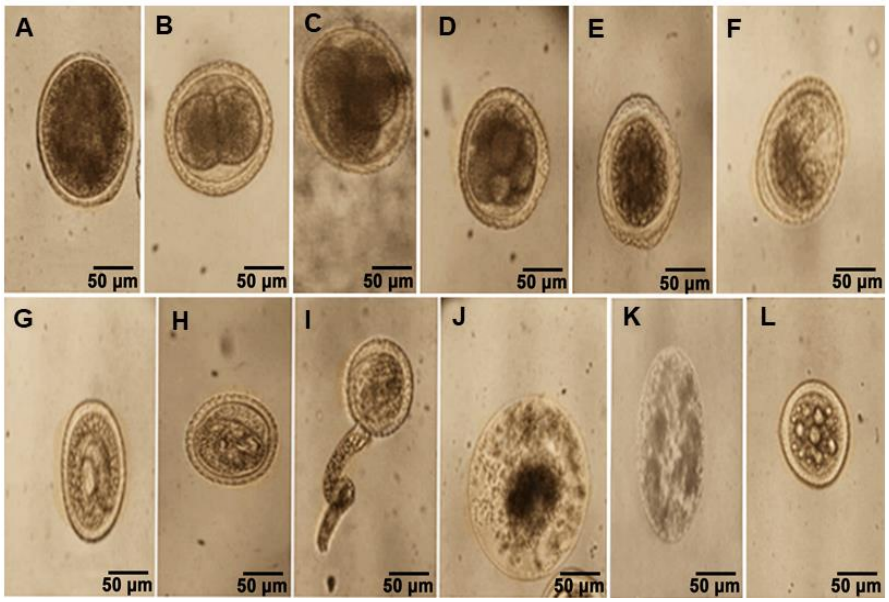

Fig. 2: Developmental stages of *Toxocara vitulorum* eggs. Eggs were incubated in 0.1N H_2SO_4 at 28°C for 18 days for ova development. (A) 1-cell-stage, (B) 2-cell-stage, (C) 4-cell-stage, (D) 8-cell-stage (early morula), (E) blastula, (F) gastrula, (G) pre-larva (larva-1), (H) larva (larva-2), (I) excystment, (J-L) dead/non-viable, (L) showing bubbled yolk, 40x magnification.

when compared to the untreated control one (Fig. 1B). In addition, the presence of small areas of lesions scattered over the cuticular surface of both female and male worms (Fig. 1D). The electron micrographs of 10 worms (5 males and 5 females) from the 2 independent experiments showed that adult females *T. vitulorum* were more sensitive to CUR ascaricidal effect than males.

Effects on *T. vitulorum* Surface Membrane Antigens

One week after third intramuscular immunization of mice with intact *T. vitulorum* TSAP antigens, significant IgG antibodies production was induced up to titer 1:8,000 as compared to naïve mice. Two repeat indirect ELISA tests were done to compare the binding capacity of TSAP from intact and CUR-exposed worms for antibodies directed to intact TSAP. There were no significant differences among CUR-exposed and intact TSAP of *T. vitulorum* regarding binding of immune serum antibodies diluted 1:1,000 up to 1:8,000.

Egg Developmental Stages Chart

Nine developmental stages were identified throughout the 18-day incubation in 0.1N H_2SO_4 at 28°C. One-cell, 2-cell, 4-cell, 32-cell (morula), blastula, gastrula, larva-1, larva-2 and excystment were identified as different developmental stages (Fig. 2). Examination of the incubated ova showed no cell division within the first 2 days (Fig. 2A); 2-4 cell stages started to appear on day 3 and continued for 3 days (Fig. 2B&C). By day 7, 8-cell stage embryo (morula) (Fig. 2D) and blastula (Fig. 2E) increased accompanied by disappearance of 1-4 cell stages, while few gastrula stages (Fig. 2F) began to be noticed. Pre-larva (Larval-1) embryonic stage (Fig. 2G) began to appear on day 11, forming incomplete ring (Cruz et al. 2012). On day 18, larva (larva-2) stage that forms a closed ring (Fig. 2H) began to appear. Excystation stage in which larva was currently exiting from ovum was also observed (Fig. 2I). Each stage was seen during 3 to 5 days with an overlap in stages. Dead ova were recognized as 3 shapes,

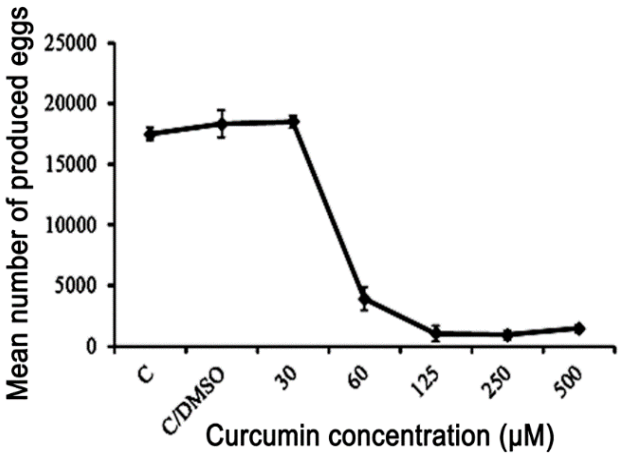

Fig. 3: Effect of curcumin on adult female *Toxocara vitulorum* egg production. *Ex vivo* adult female worms were incubated for 4hr with curcumin concentrations of 0 (controls, C; solvent controls, C/DMSO) to 500µM at 37°C then in D-PBS for 24hr at 28°C. Eggs released were collected and counted. Each point represents the mean number of eggs/4 females/experiment produced after curcumin exposure. Vertical bars depict SD around the mean.

poorly defined structure (Fig. 2J), contraction with loss in the outer membrane (Fig. 2K) and vacuolization of the cytoplasm (degenerated or bubbled yolk) throughout the incubation period (Fig. 2L).

Ovocidal Curcumin Effects

Indirect Curcumin Effect on Spawned Eggs

Eggs produced by controls (C and C/DMSO) and CUR-exposed female roundworms (30-500 µM) at 37°C for 4 hr, were collected and counted. The numbers of produced eggs/4 females were significantly decreased ($P<0.005$) with increase in CUR concentrations as compared to control groups (Fig. 3).

Eggs produced by control and 4hr CUR-exposed females (30, 60, 125, 250, and 500µM) were incubated in 0.1N H_2SO_4 for 18 days at 28°C. By repeat observations over 18 days, developmental stages starting from 1-cell embryo ending with larva-2 stage were recorded (Fig. 4). On day 4, the number of dead (ova that did not progress in development, had a dark-oval disfigured structure, and/or contained bubbled yolk) ($P<0.05$) in 30-60µM and $P<0.0001$ in 125-500µM) and un-embryonated eggs were significantly increased ($P<0.05$) in 125 and 500µM) in eggs produced by CUR pre-exposed females (Fig. 4A). After 7 days of incubation, gastrula stage was seen in eggs produced by control and 30µM CUR pre-exposed females but not in the other groups. Dead and un-embryonated eggs were significantly increased in eggs produced by 60-500µM CUR pre-exposed females (Fig. 4B). By day 11, 40% of eggs produced by CUR pre-exposed females (60-500µM) were dead ($P<0.0001$) with a significant decrease ($P<0.001$) in gastrula formation as compared to control group (Fig. 4C). By day 18, the dead eggs were significantly increased ($P<0.0001$) to 50% in eggs produced by CUR pre-exposed females (60, 125, 250, and 500µM CUR) and the embryogenesis was arrested in gastrula stage. Conversely, larval development reached 40% in eggs produced by control and 30µM CUR pre-exposed females (Fig. 4D).

Direct Curcumin Effect on Eggs

Eggs were directly exposed to different CUR concentrations (0, 30, 60, 125, 250, and 500µM) for 4hr, then washed, re-suspended and incubated in 0.1N H_2SO_4 for 18 days at 28°C. Repeat observations were done on day 4, 7, 11, and 18 to detect the developmental stages of eggs until the infected larval-2 stage (Fig. 5). On day 4, CUR pre-exposed eggs showed a significant increase ($P<0.05$ in 30-60µM and $P<0.0001$ in 125-500µM CUR) in dead and un-embryonated eggs percentages (Fig. 5A). Gastrula stage was seen in all incubated eggs on day 7, and the number of dead eggs was significantly increased ($P<0.0001$) in 125 and 500µM CUR pre-exposed eggs (Fig. 5B). By day 11, 20% of CUR pre-exposed eggs (125, 250, and 500µM) were dead ($P<0.0001$). Otherwise, gastrula stage was detected in all incubated groups. Thirty and 60µM CUR pre-exposed eggs continued their developmental to reach larva-1 stage similarly to the control group (Fig. 5C). By day 18, about 30% of CUR pre-exposed eggs (125-500µM) were dead ($P<0.0001$), showing bubbled yolk. Embryogenesis was arrested in larva-1 stage and not completed to the infected larva-2 stage, which reached 40% in control and 30µM CUR pre-exposed eggs. Sixty micromolar CUR pre-exposed eggs showed 50% reduction in the formation of the infective larva-2 stage ($P<0.0001$) (Fig. 5D).

In conclusion, direct and indirect CUR exposure to different concentrations (60, 125, 250 and 500µM) delayed *T. vitulorum* egg development and significantly ($P<0.0001$) prevented maturation to the infective stage.

DISCUSSION

Despite thousands of research papers and hundreds of preclinical and clinical studies, CUR is still not used as a drug for the multitude of inflammatory, neoplastic, neurodegenerative, microbial, and parasitic afflictions it may manage, treat, and/or prevent (Kumar and Sakhya 2013; Nelson et al. 2017a; Ragab et al. 2020; Degla et al. 2022). The main reasons counteracting CUR development as a lead drug are poor solubility and remarkable instability in aqueous solutions, including blood. These characteristics, however, would never hinder the expression of CUR biological and beneficial activity in the intestine. CUR is traditionally used for elimination of worms (Kumar and Sakhya 2013); yet, few reports have addressed CUR use in the control of intestinal parasites, namely ascarids (Bazh and El-Bahy 2013; Nasai et al. 2016). To fill such a critical gap in ethnopharmacology, a program was launched to aim at the development of CUR as a lead drug for human and animal ascarids, and started with assessing, for the first time, CUR ascaricidal effects on *ex vivo T. vitulorum* adult worms and eggs.

The roundworms freshly collected from the intestine of calves were still alive after 4hr incubation with CUR at 37°C; yet, their viability, mobility and contractility were decreased in dose dependent manner, similarly to the blood flukes, *Schistosoma mansoni* and *Schistosoma haematobium* (Abou El Dahab et al. 2019). The exact action mechanism of CUR on helminthes has not been delineated. It may involve disruption of outer membrane integrity, modulation of key enzymes activity, or interference with the function of virulence factors or

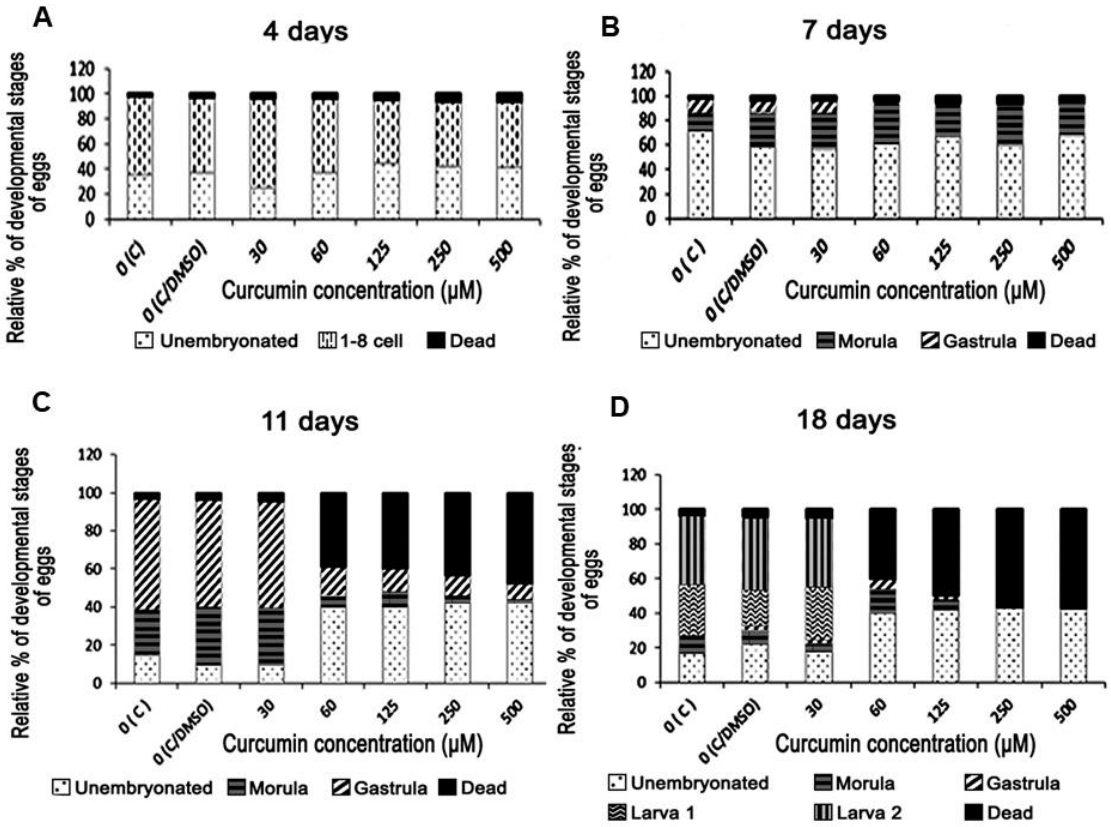

Fig. 4: Indirect effect of curcumin on *Toxocara vitulorum* ova development and embryogenesis. One thousand eggs produced by 0 (controls, C; solvent controls, C/DMSO) to 500 µM curcumin exposed adult females for 4 hr at 37°C were randomly sampled, then incubated in 0.1N H_2SO_4 at 28°C for 18 days. The relative proportions of egg developmental stages are shown after (A) 4, (B) 7, (C) 11, and (D) 18 days.

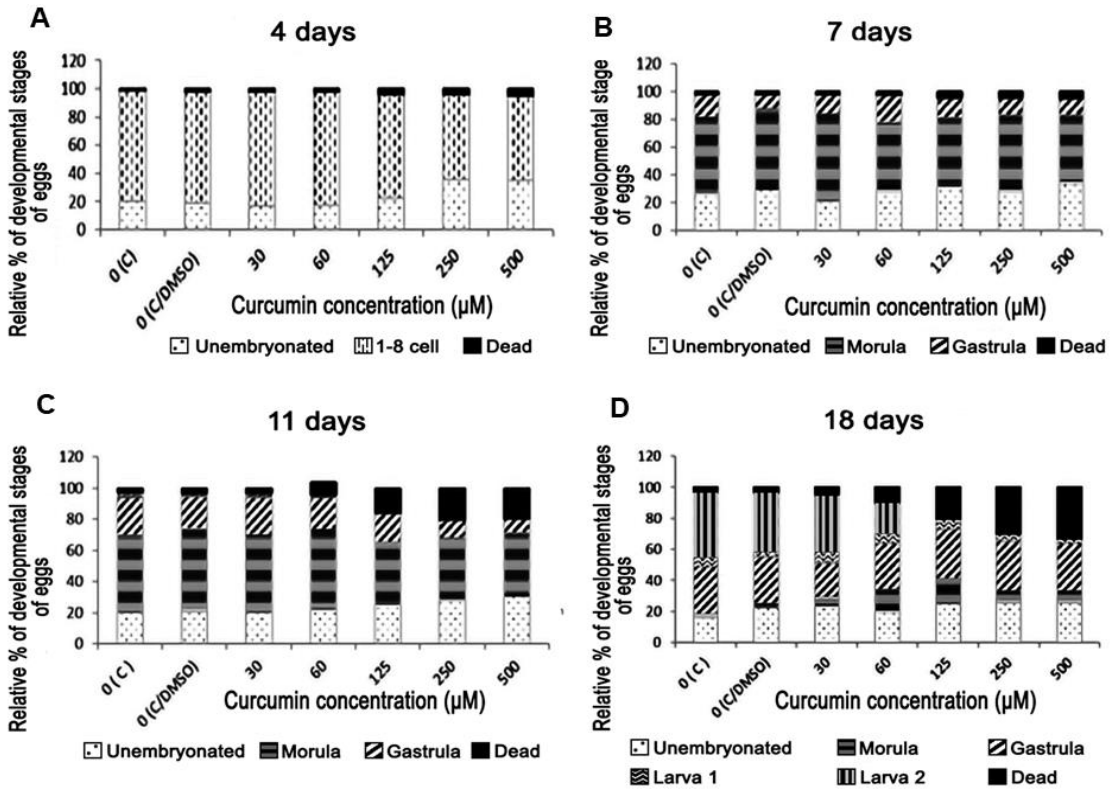

Fig. 5: Direct effect of curcumin on *Toxocara vitulorum* ova development and embryogenesis. One thousand eggs were randomly sampled following 4 hr incubation with curcumin concentrations of 0 (controls, C; solvent controls, C/DMSO) to 500µM, at 37°C, washed and incubated in 0.N H_2SO_4 for 18 days at 28°C. The relative proportions of egg developmental stages are shown after (A) 4, (B) 7, (C) 11, and (D) 18 days.

specific receptors, as CUR possesses outstanding lipophilicity, major physicochemical determinant for a drug to reach a therapeutic concentration in the target parasite (Alvarez et al. 2007).

The structure and metabolic activity of ascarid cuticle are specialized for selective absorption of nutrients and osmoregulation. Thus, destructive changes and deformation of the cuticle would lead to the parasite attrition (Alvarez et al. 2007). Ascarids use mouth parts (lips) to fix to intestinal mucosa; hence, any changes in mouth parts would lead to immediate expulsion. Scanning electron microscopy was used in the present study to determine, for the first time, the morphological changes observed in the cuticle and mouth parts of *T. vitulorum* treated with CUR. Remarkable alterations and deformity in the cuticle were apparent, and CUR concentration-dependent. The nematode *Trichostrongylus colubriformis* adult worm exposed, *in vitro*, to walnut extract rich in condensed tannin showed cuticular changes along with longitudinal and transverse wrinkles (Hoste et al. 2006). Longitudinal and transversal folds, thicker cuticular ridges, and changes in the buccal capsule were observed by Martínez-Ortíz-De-Montellano et al. (2013) upon SEM observation of adult *Haemonchus contortus* after *in vitro* contact with plants rich in tannins. Of note, our SEM results regarding the impact of CUR on *T. vitulorum* concord with the effects of the methanolic extract of *Balanites aegyptiaca* fruits (Shalaby et al. 2012).

The intact TSAP-immunized mice produced IgG antibodies against TSAP/intact that readily bound to TSAP from intact and CUR-exposed worms up to 1:8,000 dilution. The considerable production of IgG antibodies in mice sera against *T. vitulorum* antigens was also observed by Gazzinelli-Guimarães et al. (2018). Antibody binding of *T. vitulorum* CUR-treated and intact TSAP to antibodies directed to the intact antigens hardly differed, reflecting limited CUR effect on the antigenicity of the worms' surface membrane molecules. Longer worm exposure to CUR could have modified the TSAP properties because it was reported that CUR interaction with cell membranes modulates the host lipid bilayer properties, and alters the membrane protein function (Ingolfsson et al. 2007; Hung et al. 2008; Baker 2017).

Parasite egg embryogenesis is critical for transmission, spread and pathology of parasitic worms. Hence, CUR *in vitro* ovocidal efficacy against *T. vitulorum* eggs was evaluated in the current study. El Garhy and Mahmoud (2002) examined the *in vitro* effect of 6 traditional herbs on ascarid eggs and larvae. To our knowledge, the effect of CUR on *T. vitulorum* eggs was not elucidated up till now.

A total of 9 developmental stages were observed in the current study starting from 1-cell to the larva-2 stages during 18 days incubation with 0.1N H_2SO_4 at 28°C. The morphological changes of ascarid eggs under the same conditions were described (Cruz et al. 2012; Shalaby et al. 2012; Schmitz et al. 2016; Abou-El-Naga 2018). Thus, we all agreed that the eggs had developed into larva-1 by day 11. By day 18 of incubation, all larvae-1 were at larvae-2.

Following *in vitro* CUR treatment, female roundworms increasingly failed to produce eggs with increase in CUR concentration. Additionally, the produced eggs showed highly significant impairment in development up to entire failure to reach the larva-2 stage. These findings are of importance, as they suggest CUR could be used with advantage to halt transmission of ascariasis in endemic regions. In that respect, CUR could also be of importance in interfering with spread of other helminths, such as *Fasciola gigantica* (Ullah et al. 2017), and *Schistosoma mansoni* (Abou El Dahab et al. 2019).

In the current study, highly significant ($P<0.0001$) delay and interference with proper development and embryogenesis of eggs directly pre-exposed to CUR were observed and found to be CUR-dose dependent. The results indicated that CUR *in vitro* effect on *T. vitulorum* eggs is superior to the ethanolic extracts of sixteen different medicinal plants (Urban et al. 2008), and that *in vivo* studies are mandatory to elucidate CUR direct effect besides the impact of its interaction with the gut microbiota (Zhou et al. 2011; Zam 2018; Feng et al. 2019; Shen and Ji 2019).

Conclusion

The present study demonstrated for the first-time remarkable effects of CUR on *T. vitulorum ex vivo* adults and eggs. Further *in vitro* and *in vivo* studies on CUR impact on *A. suum* of pigs, *A. lumbricoides* of humans and *Toxocara* of cats and dogs are required in view of its development as a safe and cost-effective drug for control of human and animal ascariasis.

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Author's Contribution

NM and MA designed and supervised the research. Acquisition of data, analysis and interpretation were done by NM, MA and AS.

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