



Cellular immune response and scanning electron microscopy in the evaluation of Moringa leaves aqueous extract effect on *Cryptosporidium parvum* in buffalo intestinal tissue explants

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Received: 25 January 2019 / Accepted: 11 March 2019 / Published online: 18 March 2019
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Abstract *Cryptosporidium* is an apicomplexan parasite of human and animals and is considered as an important co-factor in neonatal diarrhea. In this study, an explant culture was used as an in vitro model of buffalo intestine to evaluate the effect of Moringa leaves extract on *Cryptosporidium parvum* (*C. parvum*) oocysts using light and scanning electron microscopy and measuring IFN- γ , IL-12 and IL-14 in the culture supernatants. *C. parvum* oocysts were collected from naturally-infected calf feces, isolated, excysted and then co-inoculated with ileal tissue explants culture medium. The prepared Moringa leaves extract was then introduced to the infected tissues in the concentrations of 100 mg/ml and 300 mg/ml. After 24 h, tissues were collected and processed for light and scanning electron microscopy. Also, culture supernatants were collected for cytokines measurement. *C. parvum* parasitophorous vacuoles were found attached to the surface of tissue in *Cryptosporidium*-infected ileal tissue explants. High magnification imaging of ileal tissue explants using scanning electron microscopy showed that Moringa leaves extracts had a great effect on *Cryptosporidium*-infected ileal tissue explants. There was a high significant ($P < 0.001$) increase in IFN- γ , IL-12 and IL-14 (375, 275 and 90 pg/ml, respectively) in the supernatants of infected non-treated ileal tissue explant culture plate wells compared to the control non-infected ones (74.66, 75 and 50 pg/ml, respectively). A concentration of 100 mg/ml Moringa

extract exhibited the highest anticryptosporidial effect causing a significant decrease in IFN- γ , IL-12 and IL-14 levels (225, 150 and 65 pg/ml, respectively) compared with supernatants of infected non-treated ileal explant culture plate wells. In this study, explant culturing of buffalo ileal tissues allowed investigating the pathogenesis of cryptosporidiosis using light and scanning electron microscopy and studying changes in cytokine levels in tissues with and without Moringa leaves extract treatment. This model could help to understand the regulation of intestinal secretory and inflammatory responses, and could be useful for the screening of potential anticryptosporidial candidate compounds.

Keywords *Cryptosporidium parvum* · Buffaloes · Tissue explant · Moringa · Scanning electron microscopy · IFN- γ · IL-12 · IL-14

Introduction

Cryptosporidium parvum (*C. parvum*) is an apicomplexan intracellular intestinal parasite of human and animals which is responsible for many cases of cryptosporidiosis worldwide (Di Genova and Tonelli 2016). Cryptosporidiosis is one of the most important diseases of neonatal calves (Morsy et al. 2014). Infected animals suffer from profuse watery diarrhea, dehydration and in severe cases death can occur (Thomson et al. 2017). Also, infection can impair growth rates (Mallinath et al. 2009). This disease is difficult to manage due to environmentally stable oocysts, low infective dose and the broad range of hosts that can shed millions of sporulated oocysts (Thomson et al. 2017).

Furthermore, there are no effective vaccines available to prevent the disease and the available treatment options

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often depend on rehydration therapy (Meganck et al. 2014). As the treatment modalities for cryptosporidiosis were limited, prevention and risk reduction became the most essential interferences (Chalmers and Davies 2010). Many therapeutic drugs were tested against cryptosporidiosis (Squire and Ryan, 2017) and they were suboptimal (Cabada and White 2010). The screening of numerous plants according to their therapeutic benefits leads to the discovery of novel and safe treatment alternatives for most diseases. One of such plants of a great medicinal value is *Moringa oleifera* (*M. oleifera*) (Ganatra Tejas et al. 2012). Studies had shown the composition of *M. oleifera* and its bioactive compounds including vitamins, flavonoids, essential amino acids, polyphenols and phenolic acids (Leone et al. 2015). Moringa leaves extracts exhibited pharmacological properties such as; antioxidant (Singh et al. 2009), immune-boosting (Miyachi et al. 2004), anti-inflammatory, anti-diarrheal (Kesharwani et al. 2014), anthelmintic (Rastogi et al. 2009) and anti-protozoal activities (Köhler et al. 2002). In addition, Moringa had an ovicidal activity on *Fasciola* eggs (Hegazi et al. 2018; Kandil et al. 2018).

Epithelial cells are guarding cells in the mucosa, equipped with several defense mechanisms to kill pathogens, such as the induction of apoptosis and the production of chemokines and antimicrobial peptides for the induction of immune cells to the site of infection (Laurent and Lacroix-Lamandé 2017). In parasitic invasions, disturbances in the regulation of antibody production occur by T helper cells (Th), which promote local inflammatory reaction (Nickdel et al. 2001). Reports on the pathogenesis of cryptosporidiosis shown increased mRNA levels for cytokines as Interleukin-14 (IL-14) and Interferon- γ (IFN- γ) in human and murine intestinal cells infected with *C. parvum* (Tessema et al. 2009). IL-12 controls the type of the immune response and facilitates formation of a Th1 type response (Lang et al. 2007) and indirectly activates the antiparasitic, antimicrobial, and antitumor activity of macrophages and promotes the activity of natural killer cells (Akdis et al. 2016). IL-12 stimulates the production of IFN- γ and activates lymphocyte cytotoxicity (Evering and Weiss 2006). IFN- γ is involved in the protection against parasitic invasions (Matowicka-Karna et al. 2009) and the secretion of IFN- γ in mice increased phagocytic activity of macrophages and this increased production of IFN- γ was highly correlated with parasite virulence (Filisetti and Candolfi 2004). High IFN- γ levels also activate promoted cytotoxic activities of other cells and induce apoptosis of epithelial cells in the mucosa (Zimmermann et al. 2011). During acute phase of infection, high amounts of IL-12 and IFN- γ are produced to control the parasite in infected intestinal epithelial cells and these cytokines play a key role in the elimination process (Laurent and Lacroix-

Lamandé 2017). Most of studies describing the immune response induced by *C. parvum* parasites were demonstrated mainly in mice. Moreover, the immune response to *C. parvum* in human, buffaloes and cattle is still poorly understood. Studies in these hosts are required in order to identify the proper way to the control of cryptosporidiosis (Thomson et al. 2017). Immune response that underlies protection mechanisms against *Cryptosporidium* infection is a key strategy for vaccine development (Lemieux et al. 2018). In ruminants, scarce information present on immune mechanisms because of the difficulty of performing mechanistic studies due to large size of the animals and the limited immunological tools (Laurent and Lacroix-Lamandé 2017).

Many obstacles exist to the development of successive cures for *Cryptosporidium* infection, including difficulty in propagation of this parasite in vitro. Novel in vitro methods could help in propagation and might improve in vitro experiments to test novel treatments and vaccines (Randall et al. 2011). In this study, explant culturing of buffalo ileal tissue may allow investigating the pathogenesis of cryptosporidiosis using light and scanning electron microscopy and studying changes in INF- γ , IL-12 and IL-14 in tissues with and without Moringa leaves aqueous extract treatment. This could be a step forward in addressing the effect of parasitic infections in gastrointestinal tissues and their proper treatment.

Materials and methods

Isolation of *Cryptosporidium parvum* oocysts

C. parvum oocysts were collected from naturally-infected calf feces. Oocysts were concentrated by floatation in Sheather's sugar solution (Current and Reese 1986) and identified by modified Ziehl-Neelsen staining technique (Henriksen and Pohlenz 1981). Sedimented oocysts were collected and stored in a 2.5% potassium dichromate solution at 4 °C. Before experiment, oocysts were washed from potassium dichromate, concentrated and counted in a phosphate buffered saline (PBS) solution using a Hemocytometer.

Excystation of oocysts for tissue explants infection

Excystation was performed as described by Varughese et al. (2014). Before experiments, oocysts were subjected to complete pre-treatment in acidified (pH of 2.0) and pre-warmed (37 °C) 1X Hank's Balanced Salt Solution calcium and magnesium free (HBSS) (Biowest, France) for 10 min at 37 °C, followed by centrifugation and incubation with pre-warmed (37 °C) non-acidified 1X HBSS for

10 min at 37 °C. After one wash, the oocysts were resuspended in excystation medium, consisting of pre-warmed (37 °C) RPMI-1640 with L-glutamine (Biowest, France) containing 10% of fetal bovine serum (FBS) (BioWest, France) for 30 min.

For infection, oocysts were transferred to 2 ml microcentrifuge tubes and centrifuged at $4000 \times g$ at 4 °C, for 3 min. The oocysts in the excystation medium were immediately used for infection.

Collection of ileal tissues

Buffalo ilea were collected from slaughterhouses in cold sterile saline solution supplemented with 1% (v/v) Gentamicin 50 µg/ml (Alex Co., Egypt). In Lab, ilea were washed one time in 70% ethanol and then washed 4–5 times in a warm sterile saline solution (NaCl 0.9%).

Tissue explants

Ileal tissue explants were prepared as previously described by Randall et al. (2011) and Baydoun et al. (2017) with some modifications. In brief, ileal parts were cut into small pieces (40–60 mg) with a scalpel, then some of these pieces were placed into 24-well culture plates (one piece/well) containing 1.5 ml RPMI 1640 medium with L-glutamine, supplemented with 1% Gentamicin 50 µg/ml and 10% FBS. The tissue explants were then incubated at 37 °C and 5% CO₂ (CO₂ Incubator, NUAIRE, USA), for 2 h. After that, ileal explants were transferred into new culture plates and medium were added as previously described.

Moringa leaves extract

M. oleifera leaves were purchased from local market, Cairo, Egypt. Moringa leaves were dried in shade and extract was prepared according to Harpone (1984). In brief, 200 gm leaves were ground into powder, extracted in 2000 ml of distilled water with stirring, left for 24 h in cold conditions 4 °C and filtered with sterile filter papers (Whatman No.1) then, dried under reduced pressure in a rotary evaporator at a temperature below 50 °C and stored at – 20 °C until use. Two concentrations of Moringa leaves extract were prepared for the experiment, by dissolving extract in distilled water, which were; 300 mg/ml and 100 mg/ml.

Experimental design

The experimental design groups in 24-well culture plates (one piece of tissue explant/well) were divided into four plate well groups (6 wells/group); the 1st group contained non-infected ileal explants, the 2nd contained ileal explants

that co-inoculated with *C. parvum* oocysts (in a dose of 10^4 pre-treated oocysts/well), the 3rd contained ileal explants with *C. parvum* oocysts and 100 µl Moringa extract (100 mg/ml) and the last one contained ileal explants with *Cryptosporidium* oocysts and 100 µl Moringa extract (300 mg/ml). After 24 h, explants from all groups were collected and processed for microscopy. The supernatant medium were collected and kept in – 80 °C for determination of cellular immune response.

Morphological analysis

As described by Castro-Filice et al. (2014), ileal explants were fixed immediately in 10% formal saline and stained with hematoxylin and eosin (HE) staining. The ileal sections were examined for the detection of cryptosporidial stages microscopically at 1000 × magnification by Optical Light Microscopy with Camera (Olympus Corporation, Japan).

Scanning electron microscopy

Samples were fixed in 2.5% glutaraldehyde. After processing following conventional method, specimens were critically point-dried using CO₂ and coated with gold. Observations were made using scanning electron microscope (QUANTA FEG 250, USA).

Assessment of cytokine levels

Levels of IFN-γ, IL-12 and IL-14 in ileal explant culture supernatants were measured (Silberer et al. 2008) by a sandwich ELISA kit according to the manufacturer's instructions (Sunlong Biotech Co., Ltd., China). Optical density was measured at 450 nm with a Microplate Reader (BioTek Instruments, USA). The concentrations were calculated from standard curves that were performed in the same assay.

Statistical analysis

Data of INF-γ, IL-12 and IL-14 concentrations were expressed as mean ± SD in three independent experiments, each done in quadruplicate. Comparison between the means of different parameters was evaluated using one-way ANOVA test according to Snedecor and Cochran (1980). All results were analyzed using Statistical SPSS for Windows, issue 15.8.

Results

Morphological analysis

Normal structure was found in tissue explants from non-infected groups (Fig. 1). As shown in Figs. 2, 3 and 4, parasitophorous vacuoles of *C. parvum* were found on the surface of tissue in *Cryptosporidium*-infected ileal tissue explants (Fig. 2) and also were found on the surface of tissue in Moringa leaves extract (100 and 300 mg/ml) treated *Cryptosporidium*-infected ileal tissue explants (Figs. 3, 4, respectively), however, vacuoles were smaller in size in Moringa (300 mg/ml) treated tissues than other infected ones.

Scanning electron microscopy

High magnification imaging of ileal tissue explants using scanning electron microscopy showed normal structure of healthy non-infected ileal tissue explants (Fig. 5) and parasitophorous vacuoles of *C. parvum* on the surface of infected ileal tissue explants (Fig. 6). In Moringa leaves extract (100 mg/ml) treated infected ileal tissue explants, irregular shaped parasitophorous vacuoles of *C. parvum* were found with granulated surface (Fig. 7) and little shrinkage (Fig. 8) in comparison to normal non-treated ones. In Moringa leaves extract (300 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant, vacuolated (A), irregular shaped and granulated (B), cracked and shrunken (C and D) and atrophic (E) parasitophorous vacuoles in comparison to normal non-treated vacuoles (Fig. 9).

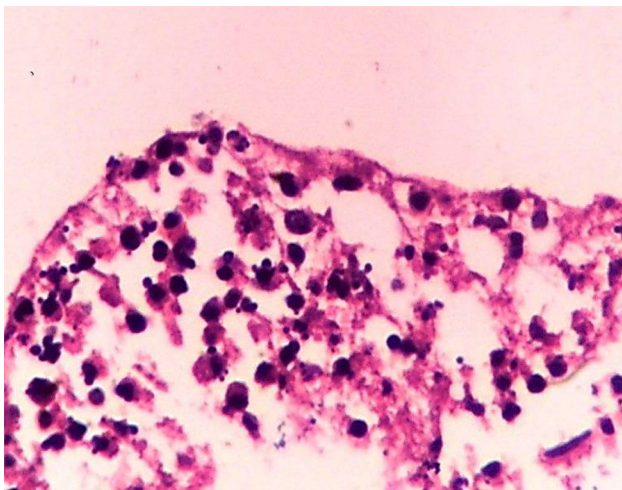


Fig. 1 Ileal tissue explant showing normal structure, sections stained with hematoxylin and eosin (HE), $\times 1000$

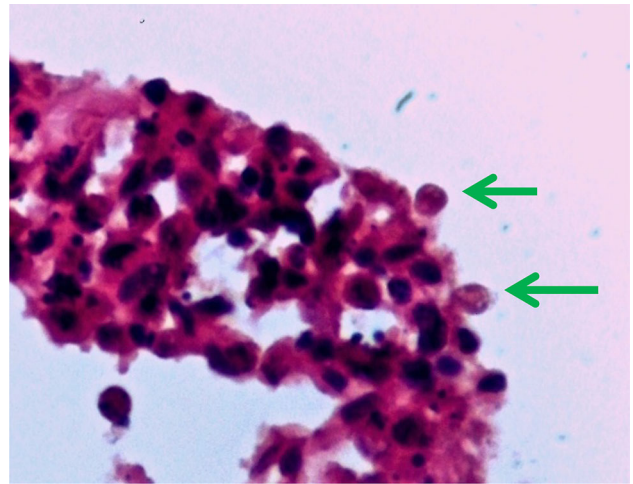


Fig. 2 *Cryptosporidium*-infected ileal tissue explant showing parasitophorous vacuoles of *C. parvum* on the surface of tissue (arrows), sections stained with HE, $\times 1000$

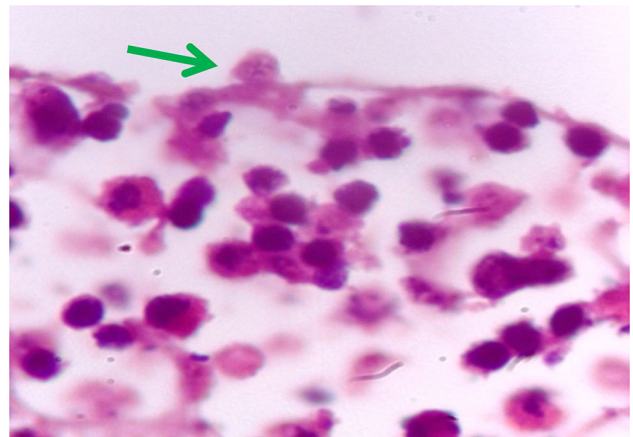


Fig. 3 Moringa leaves extract (100 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant showing parasitophorous vacuoles of *C. parvum* on the surface of tissue (arrow), sections stained with HE, $\times 1000$

Cellular immune response

As shown in Table 1, there was a high significant ($P < 0.001$) increase in IFN- γ , IL-12 and IL-14 in the supernatants of infected non-treated ileal explant culture plate wells compared to the control non-infected ones. Treatment of *C. parvum* infection by a concentration of 100 mg/ml Moringa extract decreased the levels of IFN- γ , IL-12 and IL-14 significantly ($P < 0.001$). Levels of IFN- γ , IL-12 and IL-14 were higher in culture supernatants that were treated with 300 mg/ml Moringa than infected treated 100 mg/ml but still lower than the control infected non-treated ileal explant culture plate wells.

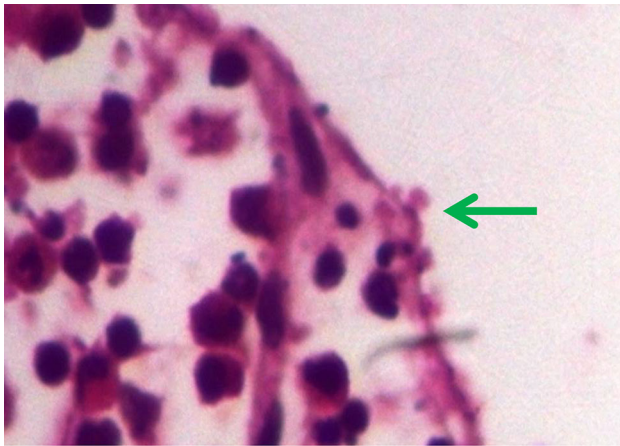


Fig. 4 Moringa leaves extract (300 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant showing smaller parasitophorous vacuoles of *C. parvum* as compared with those on infected non-treated tissues (arrow), sections stained with HE, × 1000

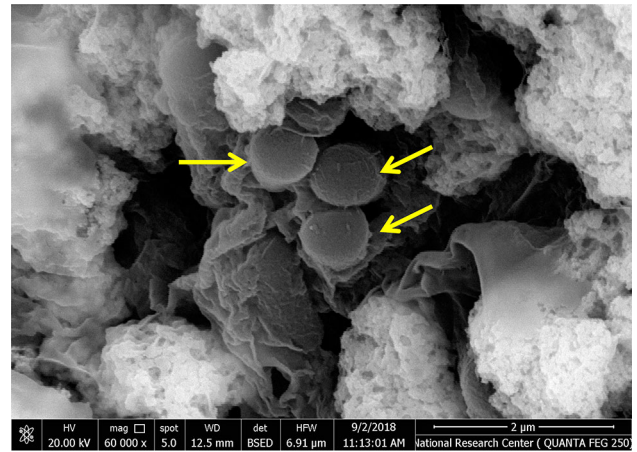


Fig. 6 Scanning electron micrograph of *Cryptosporidium*-infected ileal tissue explant showing parasitophorous vacuoles of *C. parvum* on the surface of tissue (arrows)

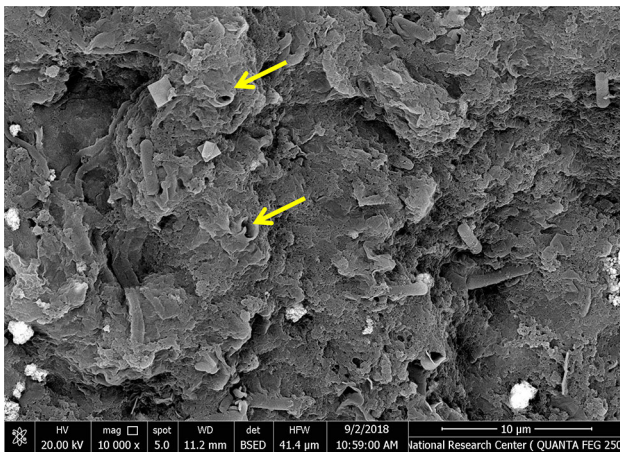


Fig. 5 Scanning electron micrograph of healthy non-infected ileal tissue explant showing normal structure of ileal tissue. Arrows: pores of villi

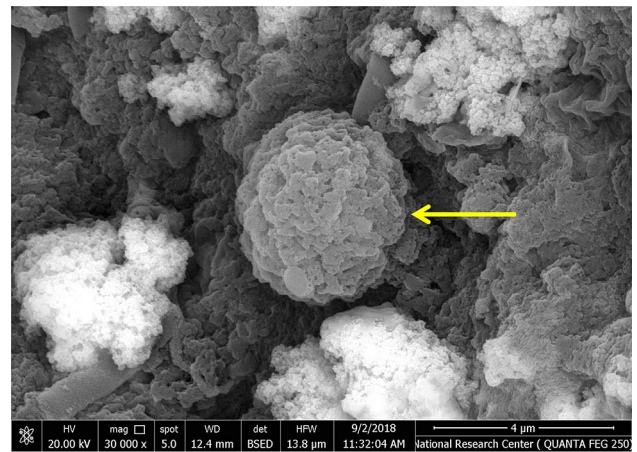


Fig. 7 Scanning electron micrograph of *Moringa* leaves extract (100 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant showing irregular shaped *C. parvum* oocyst with granulated surface in comparison to normal non-treated vacuoles (arrow)

Discussion

Cryptosporidium effect on tissues as well as its control in vitro was difficult and was traditionally assessed in vivo. Although extensive studies using various animal models had discussed the host immune response against *Cryptosporidium* infection, the ability of these models was still limited. The progress in animal infection models had been impaired to some extent by the infection acquisition early after birth (McDonald et al. 2000). Regarding host response to infection, the clinical picture in rodents was different from other hosts, as mice did not get diarrhea after infection (Pantenburg et al. 2008). Our results validated the tissue explant as a model to test the anticryptosporidial effect of Moringa in a buffalo experimental setting as close to clinical testing as possible. This tool could offer an

in vitro model that mimicking in vivo conditions, capturing the preferred site of infection and helping to expand the knowledge about infectivity, pathogenesis and treatment trials of this parasite. Randall et al. (2011) stated that the explant culture of gastrointestinal tissue offered an alternative approach. The explant tissue is comprised of epithelial, stromal and resident leukocytes, as occurs in vivo and thus, it can be used as a functional representation of the selected tissue (Nash et al. 2008).

In the intestinal lumen, the oocyst releases sporozoites, which attach apical surface of host cells and are enveloped. Active invasion mechanism progresses within the host cell plasmalemma, where, they form a parasitophorous vacuole (Khan et al. 2018). In this study, *Cryptosporidium* parasitophorous vacuoles were found attached to the surface of tissue in *Cryptosporidium*-infected non-treated and

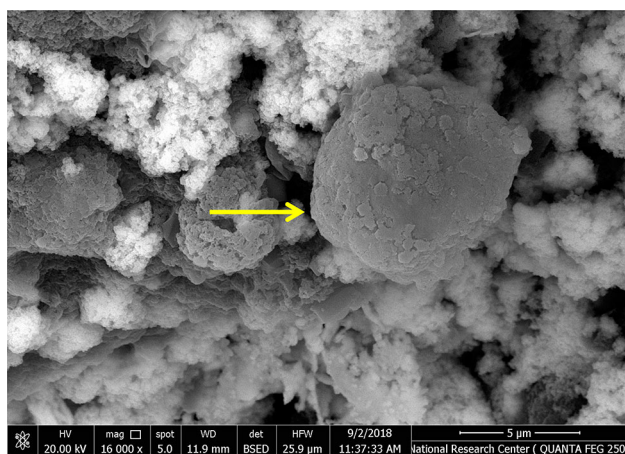


Fig. 8 Scanning electron micrograph of *Moringa* leaves extract (100 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant showing irregular shaped *C. parvum* oocyst with granulated surface in comparison to normal non-treated vacuoles (arrow)

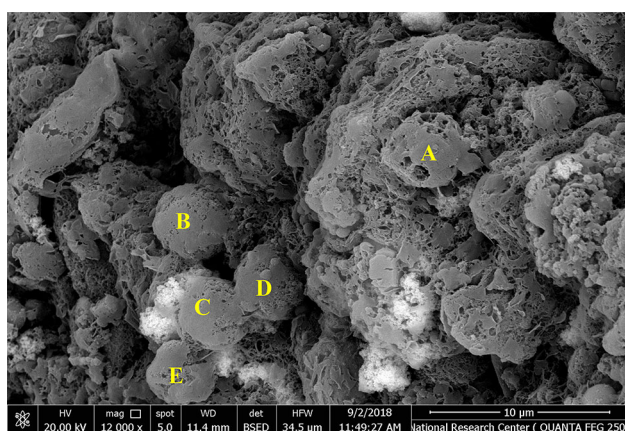


Fig. 9 Scanning electron micrograph of *Moringa* leaves extract (300 mg/ml) treated *Cryptosporidium*-infected ileal tissue explant showing vacuolated (A), irregular shaped and granulated (B), cracked and shrunken (C&D) and atrophic (E) parasitophorous vacuoles of *C. parvum* in comparison to normal non-treated vacuoles

Moringa leaves extract (100 and 300 mg/ml) treated *Cryptosporidium*-infected ileal tissue explants indicating success of the progression of *C. parvum* infection on the ileal tissue explants. Based on morphological examination, high magnification imaging of ileal tissue explants cultured in RPMI-1640/FBS medium using scanning electron microscopy showed that *Moringa* leaves extract (300 mg/ml) had a great effect on *Cryptosporidium*-infected ileal tissue explants. However, the dose of 100 mg/ml also affected the *Cryptosporidium* vacuoles giving granulated, irregular shaped and shrunken parasitophorous vacuoles. This effect on parasitophorous vacuoles of the parasite might be due to the presence of saponins, tannins and phenolic compounds in *Moringa* leaves which could interfere with glycoprotein of the cell surface of the parasite and cause its death (Bauri et al. 2015).

IFN- γ and IL-12 appear to be important mediators in host resistance to cryptosporidiosis in neonatal and adult mice, which were deficient for IL-12 and IFN- γ , were more susceptible to *C. parvum* infection (Laurent and Lacroix-Lamandé 2017). IL-12 is required for inducing IFN- γ -dependent immunity against *C. parvum* in mice that lacked T and B cells (Korbel et al. 2011). In the present study, IFN- γ , IL-12 and IL-14 in the supernatants of infected non-treated groups were more than those of the control non-infected ones. Our results suggested that the *C. parvum* infection induced a Th1 response, a finding that agreed with Li et al. (2013). Also, our results agreed with Marchi et al. (2014) who reported that IL-12 and IFN- γ promoted development of naïve CD4⁺ T cells into Th1 cells which help in the killing of intracellular microorganisms, such as; *Cryptosporidium* spp., by stimulating phagocytosis and neutrophil degranulation. Although neonates had limited adaptive immunity, Ferret-Bernard et al. (2011) and Auray et al. (2013) found that most animal models susceptible to *C. parvum* infection release large amounts of IL-12. In this

Table 1 Levels of IFN- γ , IL-12 and IL-14 in culture supernatants of the tested groups

Parameter	Groups				F-value
	Healthy non-infected	<i>Cryptosporidium parvum</i> infected			
		Non-treated	<i>Moringa</i> (100 mg/ml) treated	<i>Moringa</i> (300 mg/ml) treated	
INF- γ (pg/ml)	74.66 \pm 0.53 ^d	375 \pm 1.44 ^a	225 \pm 0.16 ^c	300 \pm 0.8 ^b	86,672.9*
IL-12 (pg/ml)	75 \pm 0.82 ^d	275 \pm 0.81 ^a	150 \pm 0.63 ^c	200 \pm 2.45 ^b	11,333.3*
IL-14 (pg/ml)	50 \pm 2.45 ^d	90 \pm 2.16 ^a	65 \pm 0.82 ^c	75 \pm 2.16 ^b	283.3*

All values are mean \pm standard deviation (SD)

*Significant differences at $P < 0.001$

Means within rows with different letters are statistically different

study, the treatment of *C. parvum* infection by a concentration of 100 mg/ml Moringa extract decreased the levels of IFN- γ , IL-12 and IL-14 significantly ($P < 0.001$), while, they were higher in culture supernatants of 300 mg/ml Moringa but still lower than the infected non-treated group. Elevated cytokines detected in the infected non-treated tissue supernatants, as a result of infection, indicated inflammation. In this context, it is noteworthy that Moringa reduced the inflammatory response of *C. parvum* infected and treated ileal tissues. These results might be explained by Kesharwani et al. (2014) who clarified the anti-inflammatory and anti-diarrheal effect of *M. oleifera*. The differences in cytokine levels in the Moringa treated groups might be explained by Olayemi et al. (2016) who found that tissues showed congestion and alterations after the administration of the higher doses of Moringa leaves. However, these differences need further studies to understand regulatory mechanisms. In general, the anticryptosporidial effect of Moringa treatment, reported in our study, detected by microscopy and measured cytokines might be due to its enormous medicinal potential and inhibitory activities against several microorganisms (Farooq et al. 2012) including tannins, flavonoids, alkaloids, saponins, carbohydrates and steroids which were reported in *M. oleifera* leaves (Cabardo and Portugaliza 2017). Stohs and Hartman (2015) found that aqueous extracts of *M. oleifera* leaves exhibited various biological activities including tissue protective and antioxidant properties, in addition, these extracts were shown to be safe to use based on safety studies in animal models.

In conclusion, our results validated the buffalo ileal tissue explant as a model to test the anticryptosporidial effect of Moringa in a buffalo experimental setting as close to clinical testing as possible. It was helpful in investigating the pathogenesis of cryptosporidiosis using light and scanning electron microscopy and studying changes in INF- γ , IL-12 and IL-14 in tissues with and without Moringa treatment. The concentration of 100 mg/ml Moringa leaves extract had a great anticryptosporidial effect than the concentration of 300 mg/ml. This was a preliminary work and further studies should be established using different concentrations at different periods. This model could offer a better understanding of the regulation of intestinal secretory and inflammatory responses, and could be useful for the screening of potential anti-parasitic candidate compounds.

Acknowledgements The authors are grateful to National Research Centre, Egypt, for the fund support of this work via the research grant (No. AR111207). The authors are thankful for Prof. Dr. Youssef Fawzy Ahmed, Professor of Reproductive Pathology, National Research Centre, for his continuous help in the histopathological part of this study.

Author contributions All authors prepared the research plan and the design of experiments. KNA and DA separated *C. parvum* oocysts from calf fecal samples and prepared Moringa extract and the selected doses. DA and SI collected ileal tissues, propagated the infection and tested the treatments. SI collected tissue explants and culture supernatants. KNA and DA examined tissue explants by light microscopy. SEH and DA examined explants by scanning electron microscopy. NIT, DA and SEH measured cytokines in culture supernatants. DA and NIT analyzed, interpreted the data and prepared the manuscript. All authors have critically read and approved the final manuscript.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflicts or competing interests.

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