In vivo effect of pomegranate (*Punica granatum*) extracts versus Nitazoxanide drug on the ileum of experimentally infected mice with *Cryptosporidium parvum* oocysts

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Abstract: Cryptosporidiosis, a major health problem, is caused by the parasite *Cryptosporidium parvum*. The current treatments for cryptosporidiosis is Nitazoxanide (NTZ). It is ineffective in some cases and there is an urgent need to search for alternatives. Alternative may be derived from natural resources. The pomegranate extract (seeds & peels) has been used effectively in traditional medicine. The purpose of this study was to compare the effectiveness of NTZ and Punica granatum seeds & peels extract as a treatment for *Cryptosporidium parvum* infection. In this study, mice were randomly divided into five groups: group I (non infected) which were subclassified into 4 subgroups mice; subgroup IA; non infected non treated, subgroup IB; non infected & treated with NTZ, subgroup IC; non infected & treated with (pomegranate seeds extract) PSE and subgroup ID; non infected & treated with (pomegranate peels extract) PPE. The other groups included Group II: (infected) with *Cryptosporidium* oocysts; Group III: (infected & treated with NTZ), Group IV (infected & treated with PSE), Group V (infected & treated with PPE). The presence of oocyst shedding, weight loss, and the histopathological changes of ileal sections were detected. At 15th and 22th dpi the production of oocysts was significantly higher in infected non treated than infected treated with NTZ, seeds extract and peels extract groups. Significant weight loss in infected non treated group (GII) as compared with group I was observed. Histopathological and ultrastructural studies of the ileum from infected group (II) showed distortion, degeneration, and atrophy of the villi. Loss of microvilli and cellular contents spilled out into the lumen. Large numbers of *Cryptosporidium* oocysts embedded in villous epithelium were observed. Histopathological study of the ileum showed improved pictures in group III & IV and more or less similar picture to control in group V. No *Cryptosporidium* oocysts were seen in the epithelium of group V. The results of the present work indicated that NTZ and *P. granatum* seeds have moderate efficacy in treatment of *C. parvum* infection. Furthermore, P. *granatum* peels exhibits higher activity as compared to seeds.
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Keywords: *In vivo;* effect; pomegranate (*Punica granatum*); extract; Nitazoxanide; drug; ileum; mice; *Cryptosporidium parvum*; oocysts

1. Introduction:

*Cryptosporidium parvum*is a parasitic protozoan that develops in the intestinal tract of humans and other mammals. It is an apicomplexan parasite that infects most vertebrates (Fayer, 2004). This parasite develops within the microvillus membrane of enterocytes causing loss of villous enterocytes and villous atrophy that lead to severe diarrhea (Liu et al., 2014).

Nitazoxanide (NTZ) is considered a thiazolide compound that exhibits broad spectrum activities against helminthes, protozoa and enteric bacteria (Hemphill et al. 2006)

Currently, the only approved drug to treat cryptosporidiosis is nitazoxanide. The efficacy of NTZ is determined by the grade of the immune system of the patient (Sparks et al., 2015). It is noticed that NTZ drughad a poor value in immunocompromised persons (Cabada and White, 2010). This necessitates the usage of other treatments.

Pomegranate (*Punicagranatum L.*) is one of the eldestidentifiedeatable fruit belonging to *Punicaceae* family (Al-Said et al., 2009). Pomegranate fruit consist of four parts: the not edible exocarp and mesocarp (peel), and also the edible endocarp which contains the seeds, creating the arils. All of these comprise remarkable bioactive particles, such as anthocyanins in the arils, hydrolysable tannins which were known to be present in greater quantity in the peel and mesocarp, punicic acid in the seeds and

others as phenolic acids & flavanols. Therefore, pomegranate whole fruit extracts are also very interesting as nutritional enhancements and nutraceuticals (Viuda-Martos et al., 2010 & Akhtar et al., 2015).

Intake of pomegranate fruit has exaggerated due to its importance in supporting health by decreasing the threat of atherosclerosis, diabetes, malignancy and neurodegenerative disorders. Some studies have shown that these compounds may be scavengers of reactive species, thus demonstrating antioxidant action (Fawole et al., 2012 & Fischer et al., 2013). Additionally, research studies have shown that pomegranate extracts shortens the duration of diarrhea and restores weight again in infected persons (Weyl-Feinstein et al., 2014).

Punica granatum (*p. granatum*) is broadly used in various states as a therapeutic agent against a multiplicity of pathogenic microorganisms. *Punica granatum* also, has anti-protozoan activities (Vijayanand and Hemapriya, 2011).

Latest years, some studies stated that pomegranate peel is a hopeful treatment for *Cryptosporidium parvum* and it is effective as anti-coccidial as well as anthelminthic beneficial agent that does not exhibit any side effects (Dkhil, 2013).

2. Materials and Methods:

Experimental animals:

Two months old, male Balb/c albino mice weighing about 25 -30 g each were used in the current study. They were housed in the animal house at Faculty of Medicine Menoufia University. Strict care and cleaning measures were utilized to keep the animal in a normal healthy condition. The animals were put in animal cages under theprevailing atmospheric conditions and also were fed to standard diet and liberal supply of tap water. They were kept under 12 h light / 12 h dark in controlled conditions of temperature (20-24ºC). All ethical protocols for animal treatment were followed.

Immunosuppression:

For immunosuppression of mice under study, the animals received synthetic corticosteroids (dexamethasone) at a dose of 0.25 mg/g/day orally for 14 successive days prior to inoculation with *Cryptosporidium* oocysts. The animals sustained to receive dexamethasone at the same dose till end of the trial (Rehg et al., 1988).

The oocysts:

Oocysts of *Cryptosporidium* were gained from naturally infected calves from butchery houses by collection of scrapings and cecal content of ileal mucous membrane (Anderson, 1985). The gathered samples were examined to approve the presence of *Cryptosporidium* oocysts by modified Ziehl–Neelsen staining method (Henriksen & Pohlenz, 1981). To preserve the infective samples, they were mixed with an equal volume of 2.5% potassium dichromate (Current et al., 1983). The infective inoculum was set as former studies. The number of *Cryptosporidium* oocysts in the stock inoculums was detected by hemocytometer (Zierdt, 1984).

Experimental infection:

All mice in the studied infected groups were orally infected with the prepared inoculums of *Cryptosporidium* oocysts on day 15 of dexamethasone treatment (Moon et al., 1982). The mice were prevented from water overnight before infection then inoculated with the prepared inoculums intra-esophageally using a tuberculin syringe connected to a polythene tube. Each mouse was given an inoculum adjusted to contain approximately 10^5 *Cryptosporidium* oocysts (Suresh and Rehg, 1996).

Drugs:

Nitazoxanide

It was purchased as a powder, formulated for oral administration (Alinia, Romark laboratories, USA) once daily oral dosein a 100 mg/kg/day starting 10 days post inoculation and lasted for 5 days later (Li et al., 2003).

Punica granatum

Pomegranate (*Punica granatum*) extracts were prepared from each peels and seeds separetly. It was purchased from local market at Giza, Egypt and was identified at Taxonomy Department, Faculty of Science, Cairo University. Voucher specimens of the plant were stored in Medicinal Chemistry Department, Theodor Bilharz Research Institute. The pomegranate fruit was washed. After that, peels and seeds were manually separated, then dried and ground to powder and stored. The plant samples powders were extracted with 85% Methanol for 24 hrs in a Soxhlet extraction apparatus. The above process repeated for three times, then pomegranate peel extract (PPE) and pomegranate seed extract (PSE) were concentrated under vacuum with rotatory Buchi evaporator to dry and collected in desiccators until been used (Rowayshed et al., 2013).

P. granatum doses of 3 g/kg body weight were prepared fresh (3 g *P. granatum* peels or seeds extracts in one ml distilled water) and administered once daily by gastric tubes (Al-Mathaland Alsalem, 2012) starting 10 days postinfection and lasts for 5 consecutive days later (Fox and Saravolatz, 2005).

Experimental design:

This study was conducted on 100Balb/c albino mice. They were classified into 5 main groups;

1- **group I (non infected)** included 40 mice which were subclassified into 4 subgroups each contained 10 mice;

subgroup IA; non infected non treated mice, received 1ml distilled water once daily by gastric

tubes, subgroup IB; non infected & treated with NTZ, subgroup IC; non infected & treated with PSE and Subgroup ID; non infected & treated with PPE with the same dose and duration as previous.

The other groups included 15 mice each:

2-**Group II: (infected)** with *Cryptosporidium* oocysts;

3-**Group III: (infected & treated with NTZ),** with the same dose and duration as previous.

4- **Group IV (infected & treated with PSE),** with the same dose and duration as previous.

5-**Group V (infected & treated with PPE)**, with the same dose and duration as previous.

Body weight measurements

All animals were weighed on 1st, 8th, 15th and 22nd day post inoculation (dpi).

Oocyst shedding:

Fresh Fecal samples were collected directly from the rectum from each animal of different groups and examined. Oocyst shedding was scored on 8th, 15th and 22nd dpi.

They were suspended in 10% formalin, homogenized and sieved then stained with modified Ziehl Neelsen staining method (Henriksen and Pohlenz, 1981). The stained fecal smear was examined microscopically through ×10 and then × 100 objectives. Then, the number of *Cryptosporidium* oocysts per mg stool from each mouse was determined.

Histopathological study:

At the end of the experiment, small parts of the ileum from each mouse was separated and divided into two parts. One part was fixed in 10% formalin, embedded in paraffin, sectioned and stained with haematoxylin and eosin stains for histopathological study (Suvarna et al., 2013).

Ultrastructure study

A small portion of the ileum was excised rapidly (within 1 min) and minced into 1x1 mm² pieces, primary fixed in 3% glutraldehyde and 0.1 M phosphate buffer at pH 7.4, post fixed in 1% osmium tetraoxide, processed and embedded in epon. Semithin sections (1 μmthick) stained with toluidine blue and examined by light microscope.

Ultrathin sections (50-80 nm thick) were contrasted with uranyl acetate and lead citrate (Al-Mathal and Alsalem, 2012) then examined with the transmission electron microscope (Seo-Russia) in Electron Microscopy Units at Faculty of Medicine Tanta University and Theodor Bilharz Research Institute.

Statistical analysis:

Results were expressed as mean ± SD. Data for multiple variable comparisons were analyzed using the one-way analysis of variance (ANOVA) followed by Turkey-Kramer's Multiple Comparison Test, where $p<0.05$ was considered significant (Armitage et al., 2001).

3. Results

Oocysts shedding:

In the current study, it was detected that at 8th dpi, there was no significant difference in the number of shedded oocysts between infected and infected & treated groups.

It demonstrated that the mean number of *Cryptosporidium parvum oocysts* were significant decreased in Group III (infected & treated with NTZ), group IV (infected & treated with PSE) & group V (infected & treated with PPE) as compared with group II (infected non-treated at 15th day post inoculation (p-value <0.05) (Table 1).

Furthermore, by 22th dpi, pomegranate peel extract treatment significantly reduced the number of *Cryptosporidium* oocysts to zero while pomegranate seed extract and NTZ reduced the number of oocysts to about 50% (Table 1).

Body weights (g).

The mean body weight of the infected/untreated GII animals showed Significant weight loss as compared to group I during the experiment ($p<0.05$) .

While non significant changes in other groups as compared to group I were observed (Table 2).

Histopathological Results:

Haematoxlyin and Eosin results

Group I (non infected)

Histological study of the ileum of this group showed long villi and invaginated crypts between the bases of villi. The villi had a core of connective tissue extending from the lamina propria. The villi were covered with enterocytes with an eosinophilic cytoplasm with basal oval nuclei and goblet cells. The luminal surface of enterocytes had a regular striated border. Some intraepithelial lymphocytes could be seen. (Fig. 1).

Group II (infected)

Histological study of the ileum from this group showed distortion, shortening, thickening, focal loss and atrophy of the villi. Also, some villi showed flatting with degeneration and shedding of epithelial cells in the lumen and sloughing of the upper tips of some villi with focal erosion.

Disruption of the covering epithelium, decreased goblet cells and cytoplasmic vacuolation were detected. Large numbers of *Cryptosporidium* oocysts embedded in villous epithelium were observed. Inflammatory cell infiltration and edema in musculosa were seen. (Figs 2 & 3).

Group III (infected & NTZ treated)

Histological study of the ileum showed improved picture with apparent increase in goblet cells. Some vacuolation in cytoplasm, some detached and irregular

villi were still seen. Decreased numbers of *C. parvum* oocysts embedded in villous epithelium were detected. Cellular infiltration and edema in musculosa was noticed. (Fig. 4).

Group IV (infected & PSE treated)

Histological study of the ileum showed improved picture with some restoration of the architecture of the crypt and villi. Apparent increase in goblet cells and some vacuolation in cytoplasm were observed. Decreased numbers of *C. parvum* oocysts embedded in villous epithelium were detected. Cellular infiltration, congested blood vessels and edema in musculosa were seen (Fig, 5).

Group V (infected & PPE treated)

Histological study of the ileum showed marked improvement with more or less similar picture to control. It was observed that some villi regained their structure and restored size of the brush border with goblet cells. Improved structure of crypts, minimal sloughing of the upper tips of few villi and vacuolation of few epithelial columnar cells were detected. Minimal edema and inflammatory cell infiltration were seen. No *C. parvum* oocysts in villous epithelium were noticed in this group (Fig.6).

Electron Microscopic Results:

Transmission electron microscopic study of the ileum of group I showed pale microfold cells (M cell) contained euchromatic oval-shaped nuclei, the intercellular junction between the M cell and the microvillus enterocytes was darker. Some apical surface of M cells plugged into the intestinal lumen. Some microfilament bundles in the apical cytoplasm were revealed. Terminal web and discontinuous basement membrane of microvillus enterocyte were detected.

Regular microvilli and mitochondria with prominent cristae were also seen. Some lymphocytes infiltrated M cell and others incorporated inside the cytoplasmic pockets. Lymphoblast with their euchromatic oval nuclei were seen. Also, Paneth cells with basal vesicular nucleus, prominent nucleolus and large secretory granules with protein core were observed. Most of the granules were electron dense surrounded with clear halos. Abundant RER and lysosomes were noticed (Figs 7,8 & 9).

An electron micrograph of ileum from group II showed loss of microvilli, interrupted intracellular junctions and cellular contents spilled out into the lumen.

Paneth cell with degenerative changes, cytoplasmic vacuolations, depleted granules, dilated Golgi, dilated vesiculated RER, lysosomes and swollen mitochondria were noticed. Lamina propria showed leukocyte, lymphocyte and macrophage infiltration. Vacuoles and edema were also seen.

At the site of degenerated tissue, many oocysts and trophozoites with altered structures were seen (Figs 10-16).

An electron micrograph of ileum from group III showed regenerated columnar epithelium with less prominent microvilli, some mitochondria, RER and dilated Golgi. Detached epithelial tissue in the lumen and some loss of tissue were still present.

Some oocysts appeared vacuolated with incomplete content, lacked internal features. other oocysts with degenerated parasitophoric envelope and malformation were seen (Figs 17-19).

Electron micrographs of ileum from group VI Showed partially regenerated columnar epithelium with less prominent microvilli, mitochondria, RER, some vacuoles were detected.

Cryptosporidium oocysts lacked internal features and had a degenerated parasitophoric envelope, oocyst with complete malformation were revealed (Figs. 20 & 21).

An electron micrograph of ileum from group V showed marked improvement, regenerated columnar epithelium with euchromatic nuclei and prominent nucleoli more or less similar to control with prominent microvilli. The mitochondria and lymphocytes surrounded by rough endoplasmic reticulum were detected. Intercellular junctions were prominent. Few vacuoles were still also present. No *Cryptosporidium* oocysts were seen in the epithelium of group V (Figs 22 & 23).

Table1: Mean (mean ± SE) oocyst shedding (oocyst number per.01 g of feces).

	Group GII			Group GIII			Group GIV			Group GV		
8th dpi	6.20	±	0.75	6.13	±	0.72	6.00	±	0.82	5.27	±	0.85
15th dpi	6.93	±	0.68	4.53	±	0.88	4.47	±	0.81	1.53	±	0.62
22th dpi	7.40	±	0.61	3.87	±	0.72	3.53	±	0.72	0.00	±	0.00

day post-inoculation (dpi)

The present study demonstrated that the mean number of *Cryptosporidium parvum oocysts* were significant decreased in Group III (infected & treated with NTZ), group IV (infected & treated with PSE) & group V (infected & treated with PPE) as compared with group II (infected non-treated at 15th and 22th day post inoculation (p-value <0.05).

Table 2: Mean (mean ± SD) body weights (g).

	Group GI	Group GII	Group GIII	Group GIV	Group GV
1st dpi	25.50±0.13	26.74±0.36	25.90±0.22	27.43±0.11	26.98±0.45
8th dpi	27.81±0.35	23.35±0.16	26.93±0.39	28.30±0.40	28.27±0.31
15th dpi	28.74±0.19	21.14±0.19	27.21±0.18	29.21±0.40	30.97±0.52
22th dpi	30.98±0.74	18.96±0.54	28.17±0.55	30.10±0.61	32.59±0.14

The mean body weight of the infected/untreated GII animals was highly significant low throughout the duration of the experiment as compared to group I (non infected) ($P < 0.001$)

Groups of infected mice that were treated with either NTZ and *P. granatum* seeds and peels (GIII, GIV & GV respectively) showed non significant weight changes during the treatment period as compared to group I (non infected) (P>0.05) (Table 2).

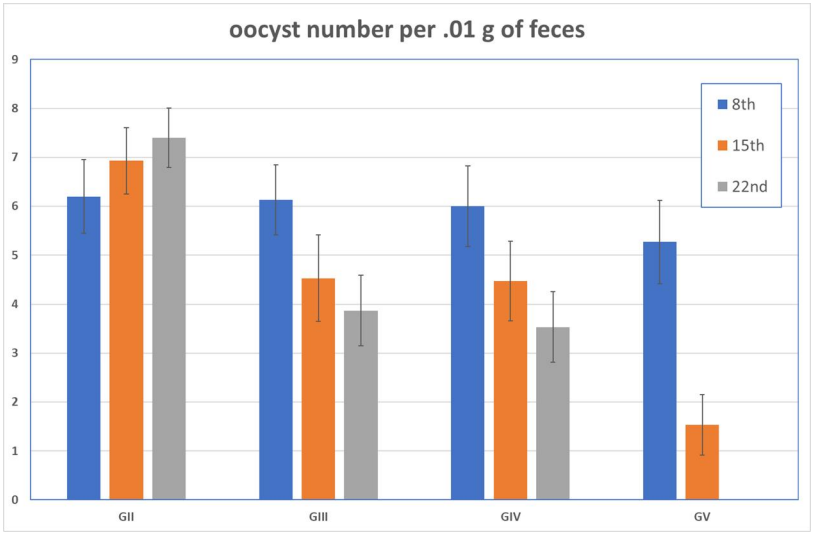

Chart 1

Chart 2

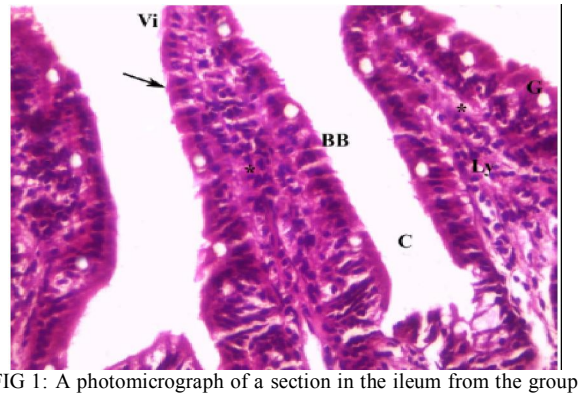

FIG 1: A photomicrograph of a section in the ileum from the group (I) showing long villi (Vi) and invaginated crypts (C) between the bases of villi. The villi have a core of connective tissue (∗) extending from the lamina propria. The villi are covered with enterocytes with an eosinophilic cytoplasm and basal oval nuclei (→) and goblet cells (G) also seen. The luminal surface of enterocytes has a regular striated border (BB). Some intraepithelial lymphocytes can be seen (Ly). H & E X 400.

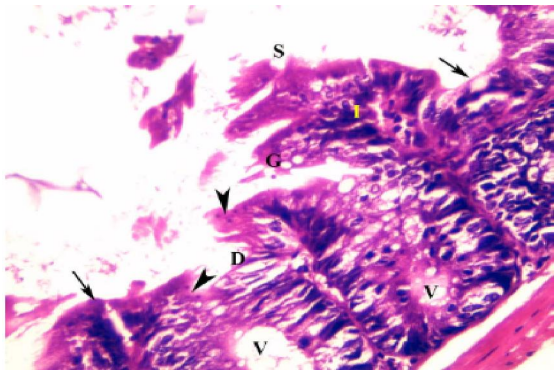

FIG 2: A photomicrograph of a section in the ileum from the infected group (group II) showing distortion, shortening, thickening, and focal loss of the villi (→). Atrophy and sloughing of the upper tips of some villi (S). Disruption of the covering epithelium (D), few goblet cells (G) and cytoplasmic vacuolation (V) as well as infiltration of lamina propria with inflammatory cells (I). Note: increased numbers of C. parvum oocysts embedded in the villous epithelium of this group (arrow head). (H & E ×400)

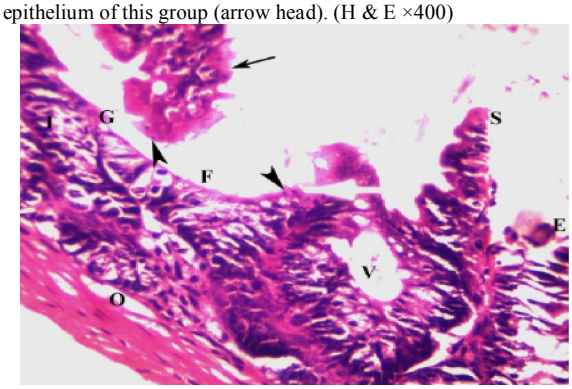

Fig 3 photomicrograph of a section in the ileum from group II (infected) showing shedding of epithelial cells in the lumen (→), cytoplasmic vacuolation (V), flatting of the villi (F), degeneration, atrophy with sloughing of the upper tips of the villi (S), focal erosion (E) inflammatory cells infiltration (I), edema in musclosa (O) and decreased goblet cell (G). Note: increased numbers of C. parvum oocysts embedded in villous epithelium of this group (arrow head) (H & E ×400)

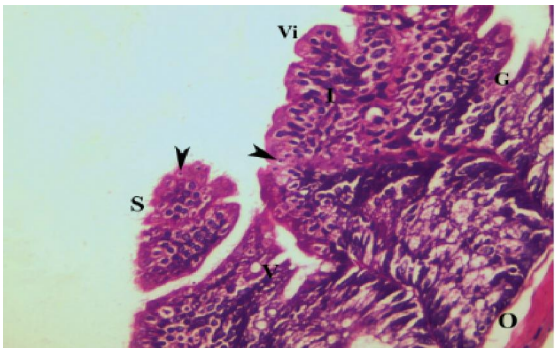

Fig 4: A photomicrograph of a section in the ileum from group III (infected & NTZ) showing improved picture with cell infiltration (I), and odema (O) in musclosa, some goblet cell (G), vacuolation (V), detached villi (S) and irregular villi (Vi). Note: Decreased numbers of *C. parvum* oocysts embedded in villous epithelium in this group of mice (arrow head) (H & E ×400)

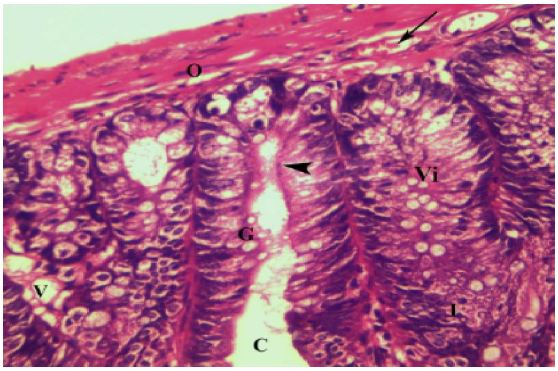

Fig 5 A photomicrograph of a section in the ileum from group IV (infected & PSE) showing some improvement with cell infiltration (I), congested blood vessels (arrow) and edema (O) in musclosa, some restoration of the architecture of the crypt) (C) and villi (Vi). Note: few goblet cell (G), vacuolation (V) decreased numbers of C. parvum oocysts (arrow head). (H & E ×400)

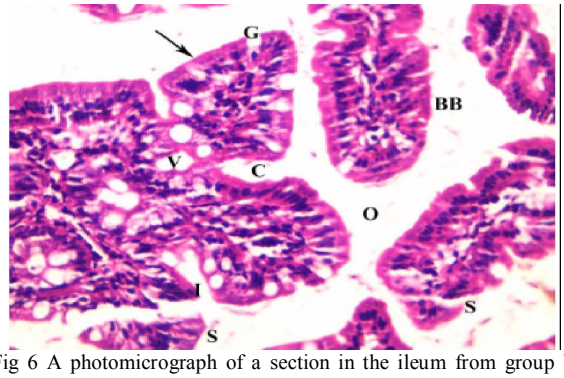

Fig 6 A photomicrograph of a section in the ileum from group V (infected & PPE) showing marked improvement with some edema (O), sloughing of the upper tips of few villi (S) and inflammatory cell infiltration (I). Some villi regained their structure (arrow). Restored size of the brush border (BB) with apparent increased goblet cells (G), improved structure of the crypt (C) and vacuolation of some epithelial columnar cells (V) were observed. No Cryptosporidium parvum oocysts could be detected in villous epithelium of this group. H & E (×400)

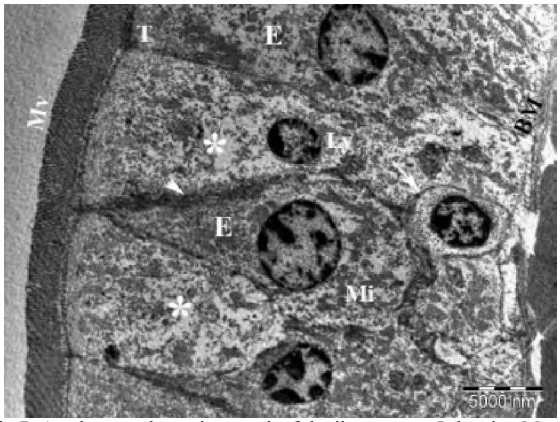

Fig 7: An electron photomicrograph of the ileum group I showing M cell (*), the intercellular junctions (arrow head) between the M cell and the microvillus enterocytes (E) are darker. Small lymphocytes (Ly) inside M cell and other incorporated inside the cytoplasmic pockets (arrow) were observed. Notice: discontinous basement membrane (BM), microvilli (Mv), mitochondria (Mi) and terminal web (T) in microvillus enterocyte. 850X

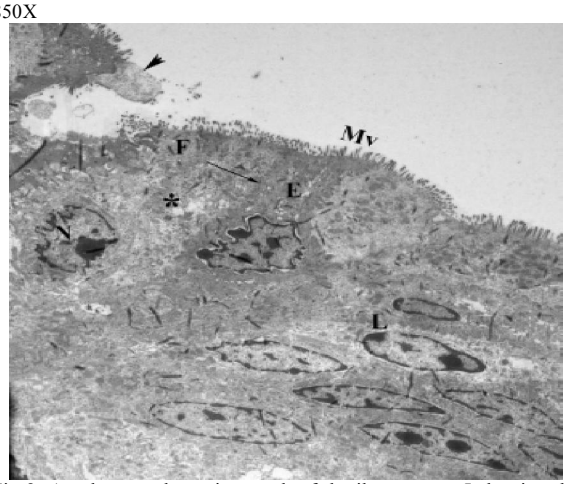

Fig 8: An electron photomicrograph of the ileum group I showing the pale microfold cells (M cell) (*), contain euchromatic oval-shaped nuclei (N). the intercellular junctional (arrow) between the M cell and the enterocytes (E), microvilli (Mv) The apical surface of M cells pulges into the intestinal lumen (arrow head), Remnants of microfilament bundles (F) in the apical cytoplasm. Lymphoblasts (L) with their euchromatinic oval nuclei. 1000X

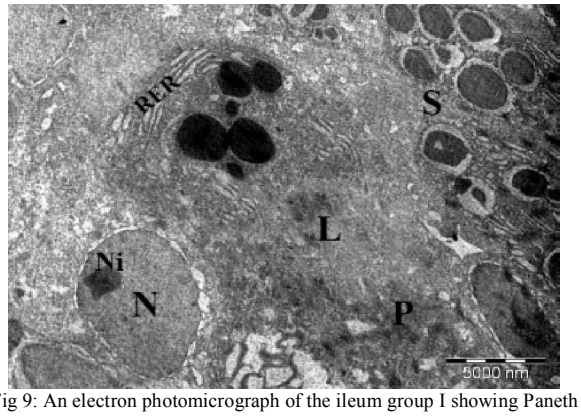

Fig 9: An electron photomicrograph of the ileum group I showing Paneth cells (P) with basal nucleus (N) and prominent nucleolus (Ni), abundant RER and large secretory granules (S) with protein core and lysosome (L) 1500X

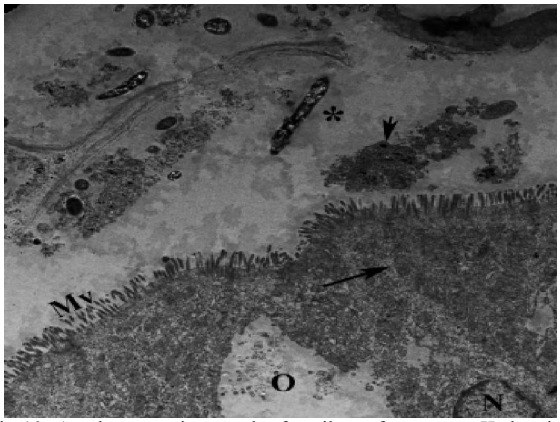

Fig 10: An electron micrograph of rat ileum from group II showing C. parvum trophozoite (*) at the site of degenerated tissue, interrupted intracellular junctions (arrows), and cellular contents spilled out into the lumen (arrow head), part of enterocyte nucleus (N) Notice: odema (O), microvilli (MV). 1500X

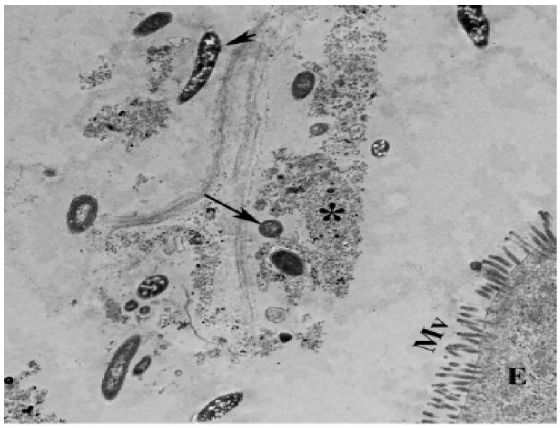

Fig 11: An electron micrograph of ileum from group II showing **oocyst (arrow) at the site of degenerated tissue,** trophozoite (arrow head) and cellular contents spilled out into the lumen (*), Notice: microvilli (MV) of enterocyte (E). 3000X

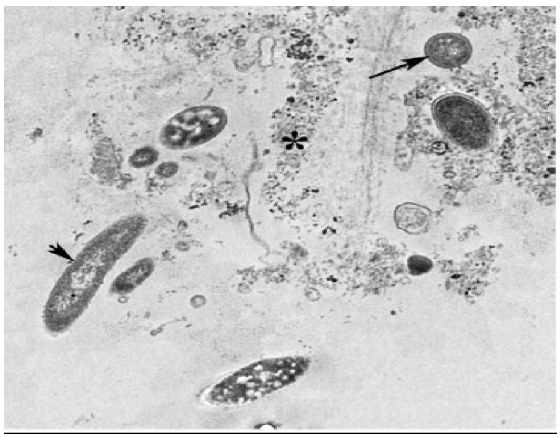

Fig 12: An electron micrograph of rat ileum from group II showing **oocyst (arrow) at the site of degenerated tissue,** trophozoite (arrow head) and cellular contents spilled out into the lumen (*), 5000X

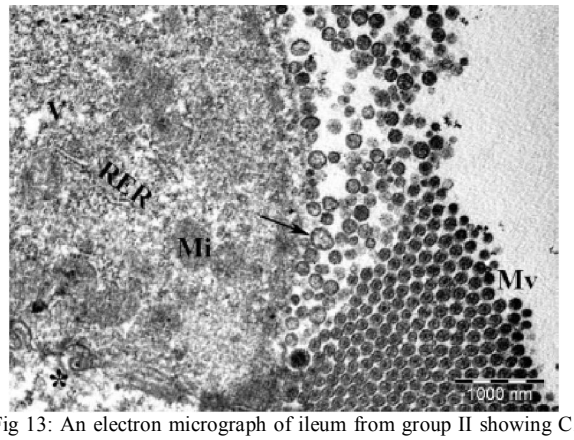

Fig 13: An electron micrograph of ileum from group II showing C. parvum oocyst (arrow) at the site of degenerated tissue, RER, interrupted intercellular junction (*) Notice: vacuoles (V), microvilli (MV), mitochondria (Mi). 5000X

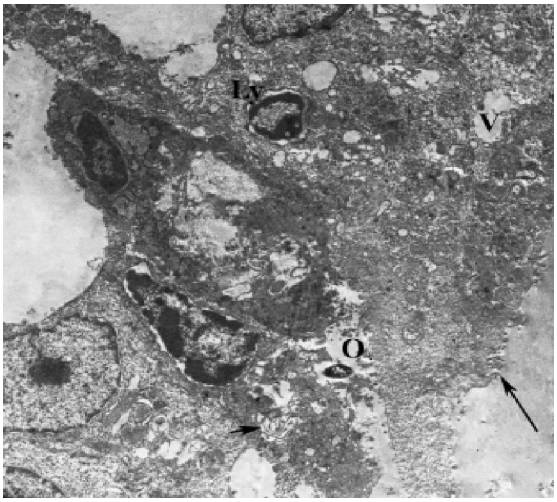

Fig 14: An electron micrograph of rat ileum from group II showing Loss of microvilli (arrow), lymphocyte (Ly), dilated Golgi saccules (arrow heads), and vacuoles (V) and edema (O) 1500X

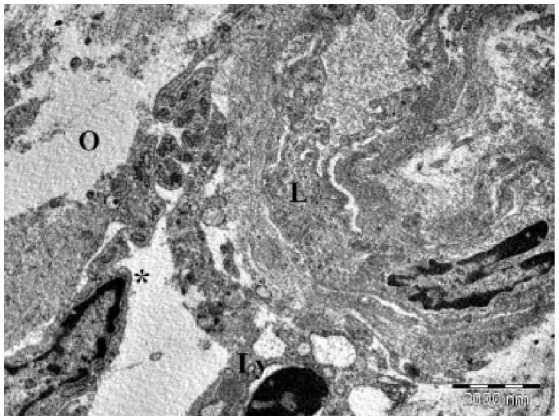

Fig 15: An electron micrograph of rat ileum from group II showing Lamina propria (L) with leukocyte infiltration, lymphocyte (LY), macrophage (*) and odema (O) 2500X

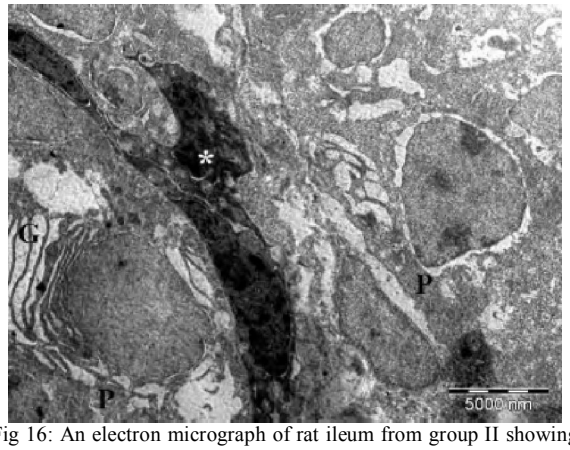

Fig 16: An electron micrograph of rat ileum from group II showing Panth cell (p) with depleted granules, dilated golgi (G) and macrophage (*) with dark nuclei. 1000X

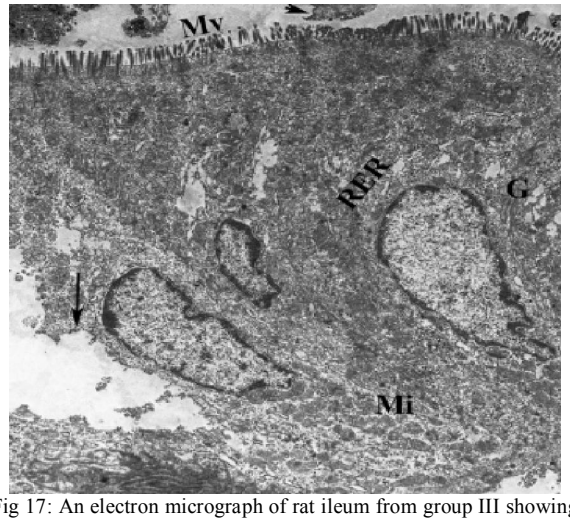

Fig 17: An electron micrograph of rat ileum from group III showing regenerated columnar epithelium with less prominent microvilli (MV), some mitochondria (Mi), rER, Notice: dilated golgi (G), detached epithelial tissue in the lumen (arrow head) and loss of tissue (arrow). 1500X

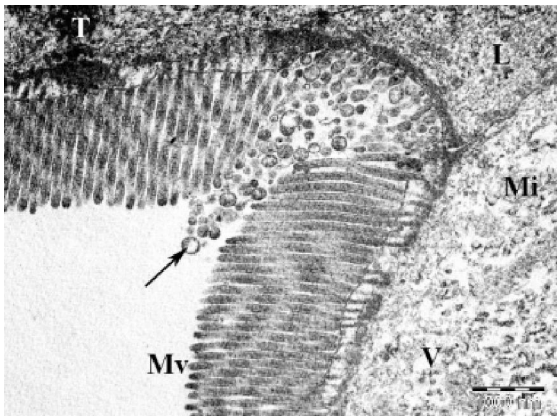

Fig 18: An electron micrograph of ileum from group III showing regenerated columnar epithelium with some microvilli (MV), less prominent mitochondria (Mi), vacuoles (V), lysosomes (L) terminal web (T) in microvillus enterocyte. Note: vacuolated oocyst with incomplete content were detected (arrow).4000X

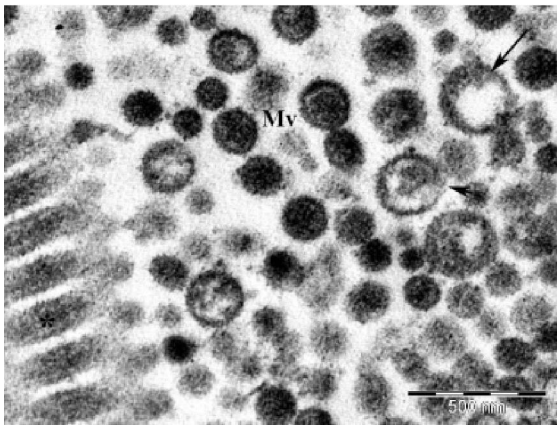

Fig 19: An electron micrograph of ileum from group III showing a parasite between microvilli (MV) that lacks internal features and has a degenerated parasitophoric envelope (arrow) with complete malformation (arrow head). 15000X

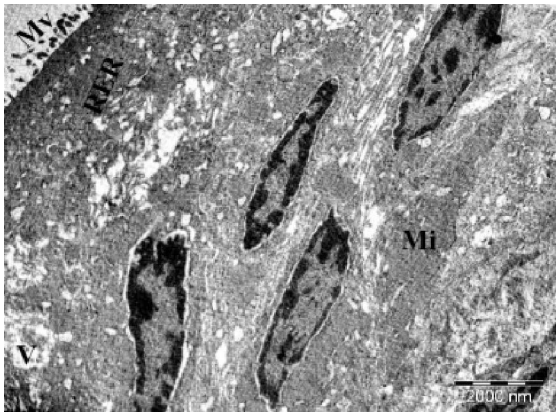

Fig. 20: An electron micrograph of rat ileum from group IV showing regenerated columnar epithelium with less prominent microvilli (MV) mitochondria (Mi), RER, some vacuoles (V). 2500X

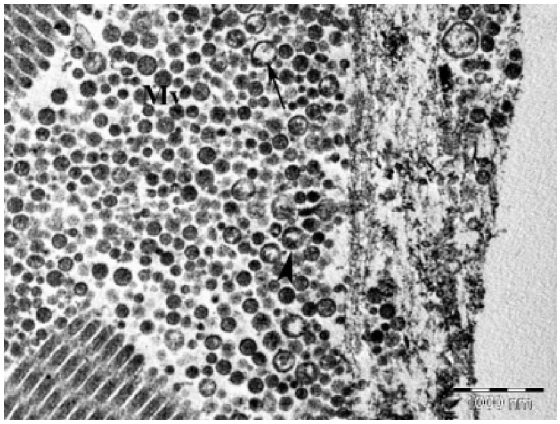

Fig 21: An electron micrograph of ileum from group IV showing a parasite that lacks internal features and has a degenerated parasitophoric envelope (arrow) and oocyst with complete malformation (arrow head) between microvilli (MV). 5000X

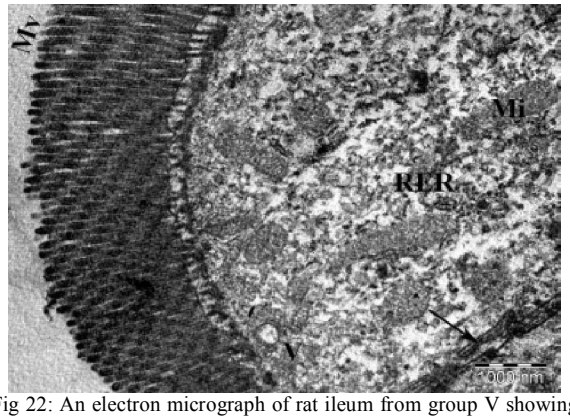

Fig 22: An electron micrograph of rat ileum from group V showing Regenerated columnar epithelium more or less similar to control with prominent microvilli (Mv). The mitochondria (Mi), rER, some vacuoles (V) were also present. Notice: intercellular junction are prominent (arrow). 3500X

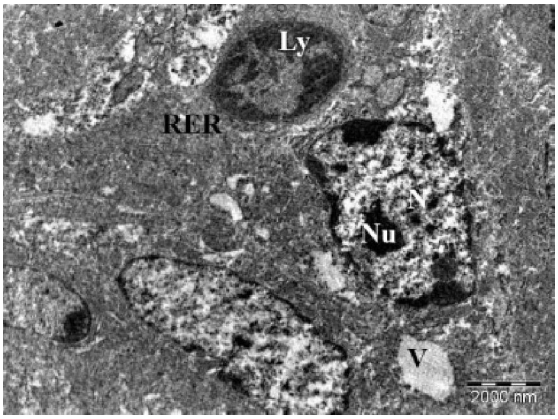

Fig 23: An electron micrograph of rat ileum from group V showing marked improvement, regenerated columnar epithelium with euchromatic nuclei (N) and prominent nucleoli (Nu) lymphocyte (Ly) surrounded by prominent rough endoplasmic reticulum (RER) Notice: some vacuoles (V) are still present. 2000X

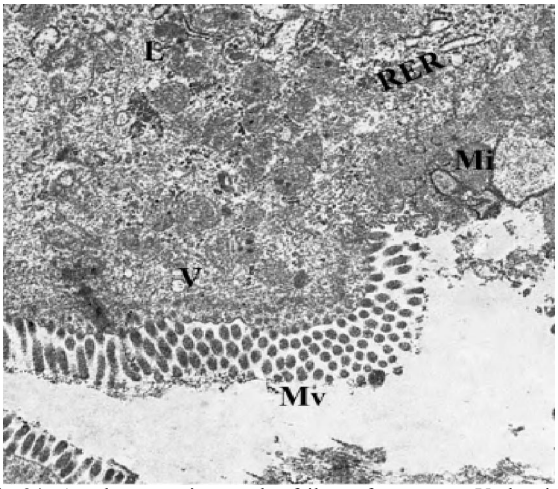

Fig 24: An electron micrograph of ileum from group V showing regenerated columnar epithelium more or less similar to control with prominent microvilli (Mv). The mitochondria (Mi), RER, some vacuoles (V) are also present. Notice: some lysosomes are seen (L). No Cryptosporidium oocysts could be detected. 5000X.

4. Discussion:

Cryptosporidium parvum is an enteric protozoan transmitted by faeco-oral route that causes unbearable diarrhea in the affected host (Sasahara et al., 2003). It is a cosmopolitan disease but more common in developing regions where poor hygiene and water pollution are predominant. In immune-compromised individuals such as those with HIV, cryptosporidiosis is actually life threatening with higher death rate (Eisenberg et al., 2001).

Oocyst shedding, body-weight and histological changes are useful for determining the severity of *C. parvum* infections (Enemark et al., 2003). Therefore, in this study, these parameters were examined during the course of *C. parvum* infections either alone or with NTZ, *P. granatum* seeds and peels treatments.

The present study demonstrated that the mean number of *Cryptosporidium parvum oocysts* were significantly high in infected non-treated group at 15th and 22nd day post inoculation. These finding were coincided with investigations carried out by Al-Mathal and Alsalem, 2012.

In addition, Certad et al., 2007 revealed that fecal *C. parvum* oocyst shedding occurred in all infected mice 7 days after inoculation. This was likely due to the compromised immunity of the infected animals (Takeuchi et al., 2008).

Infected and treated mice with NTZ showed less number of *Cryptosopridium* oocysts shedded in the stool that were in accordance with Viel *et al*., 2007 who stated reduction of oocyst output to 42 % in NTZ treated group.

Nearly, similar findings in seed treated group (IV) were observed as in NTZ treated group (III). Shedded oocyst was nil in peel extract group (V). This was in agreement with Al-Mathal and Alsalem, 2012 who reported that *C. parvum* infected mice treated with *P. granatum* peel suspension presented that oocyst shedding was very significantly reduced by day 14 pi and was totally eradicated by day 28 pi.

The reduction and elimination of fecal oocyst shedding in response to *P. granatum* treatment seen here may be attributable to direct effect on parasite growth in the intestines, production of the sexual phases, and/or the formation of oocysts. *P. granatum* contains major phenolic compounds, such as organic acids (Choi et al., 2009), that can directly inhibit *C. parvum* infections. Additionally, tannins that exist in pomegranate peels also have a strong impact on microbes (Watarai et al., 2008).

The infected/untreated GII animals showed significant weight loss throughout the duration of the experiment as compared to group I. This is in agreement with Guitard et al., 2006 who added that weight reduction is a major pathological impact of cryptosporidiosis. However, Sasahara et al., 2003 did not observe any variance in body weights.

Weight loss may be due to poor absorption arising from mucosal surface loss, chronic malabsorption and secondary infections to *C. parvum* infection (Brantley et al., 2003).

Infected mice that were treated with either NTZ and *P. granatum* seeds and peels (GIII, GIV & GV) showed non significant weight changes during the treatment period as compared to group I (non infected). This could be explained by eliminating the parasite toxic effects effect by NTZ and *p. granatum* seeds and peels respectively on *C. parvum* (Abdou *et al*., 2013).

Histopathological examination of the present study in infected group (II) revealed distortion, shortening, thickening, atrophy and sloughing of the upper tips of some villi with focal erosion. Also, some villi showed flatting with degeneration and shedding of epithelial cells in the lumen. These findings were in agreement with Guitard et al., 2006 who explained that by the toxins secreted by *C. parvum* that directly damage the epithelial cells. *C. parvum* displaces brush borders causing an asymmetrical loss of epithelial cells resulting in shortening and fusing of the villi.

Inflammatory cell infiltration and edema were seen due to parasite induced atrophy of the cells. This coincided with the work of other authors who explained that infection also stimulated lymphocytes and macrophages as a part of the host defense mechanism against the parasites (Robinson et al., 2008).

Decreased goblet cells and cytoplasmic vacuolation was observed and this was in agreement with Maruyama et al., 2007 who indicated the loss of goblet cells and some absorbent cells in the shape of vacuoles is common after *C. parvum* infection.

Light microscopic results were confirmed by transmission electron microscope examination. In group GII, *C. parvum* oocysts were detected at the site of degenerated tissue and cellular contents spilled out into the lumen. Interrupted intracellular junctions, Loss of microvilli and loss of normal architecture were also observed. Lamina propria showed leukocyte, lymphocyte and macrophage infiltration.

Paneth cells showed degenerative changes with depleted granules, dilated Golgi and dilated vesiculated RER. Vacuoles in different cells and edema in lamina propria were observed, which can be explained by the infection causing cellular atrophy. This along with inflammation in the mucosa could lead to a decrease in the absorption of water and electrolyte in the intestines (Warren et al., 2008).

A section in the ileum of group III (NTZ group) showed improved picture with some restoration of the architecture of the crypts and villi and apparent

increase in goblet cell. Some sections revealed detached irregular villi and vacuolation in cytoplasm, congested blood vessels, cellular infiltration, edema and decreased numbers of *C. parvum* oocysts as compared with infected group. This is in accordance with Pedraza-Díaz *et al*., 2001 who concluded that NTZ yielded moderate efficacy against *C. parvum* infection. These results were confirmed by electron microscopic results of NTZ group.

The increased number of goblet cells after NTZ treatment indicated that the immune response had improved in the mucosa of intestine. These cells play an important role in the anti-microbial antibodies production that may be attributed to increase in DNA and protein content (mucoprotein) in the mucosa (Schnyder et al., 2009 & Manar, 2012).

A histological section in the ileum of group IV showed improved picture nearly similar to group III and this was in coincidence with Watarai et al., 2008 who added that, organic acids in *P. granatum* have inhibitive effects on *C. parvum* infections. Additionally, the hydroxyl group of the phenolic compounds can increase toxicity against all organisms.

Furthermore, pomegranate has also anti-inflammatory properties greater than other anti-inflammatory treatments because it contains more effective and less toxic elements, including flavonoids. This is in agreement with a report that indicated the antioxidants in the pomegranate affected mammalian cells and protozoa differently (Belal et al., 2009).

An electron micrograph of the ileum from group IV confirmed histological results with some regenerated columnar epithelium. This is in harmony with Johanningsmeier and Harris, 2011 who attributed this to pomegranate seeds which are polyphenol-rich with high antioxidant capacity.

A section in the ileum of group V showed marked improvement with more or less similar picture to control. Some villi regained their symmetrical architecture. Restored size of the brush border with apparent normal goblet cells and improved structure of the crypts, minimal sloughing of the upper tips of few villi and minimal vacuolation of few epithelial columnar cells were seen. Minimal edema and inflammatory cell infiltration were noticed. No *C. parvum* oocysts could be detected (Choi et al., 2009). Therefore, *P. granatum* treatments may have both anti-parasitic and immune-modulatory effects. These data suggested that *P. granatum* treatments have positive influences on ileal tissue health in addition to its eliminatory effects on *C. parvum* infections. This improvement of the ileal tissue treated with *P. granatum* could be attributed to the remarkable decrease in the number of *C. parvum* oocysts on the brush border and stoppage of parasite production (Bourlioux et al.,2003).

An electron micrograph of the ileum from group V showed marked improvement with few vacuoles was still also present. This is in agreement with Watarai et al., 2008 who explained that by a decrease in the number of parasites because inhibitory effects of the organic acids on *C. parvum* infections.

Additionally, Choi et al., 2009 proved that pomegranate peel suspension induced severe deformation in the different stages of the parasite, causing the absence of the feeder organelle. Without this organelle, the parasites are unable to feed, which could lead to tears in the paracytophorous vacuole membrane and probably the death of the parasite. This in turn would lead to the lack of sexual stage parasites.

The difference in the antioxidant activity of the peel and seed may be attributed to their different phenolic compositions. In all, this data indicated that the pomegranate peel had a direct impact on the parasite at various stages of its development that eventually resulted in parasite decomposition and death. In addition, parasite death went hand in hand with intestinal tissue recovery as the lack of parasites allowed for the intestine to recover (Singh et al., 2002).

5. Conclusion

The results of the present work indicated that NTZ and *P. granatum* seeds have moderate efficacy in treatment of *C. parvum* infection. Furthermore, P. *granatum* peel exhibits higher activity as compared to seeds.

References

1. Abdou AG, Harba NM, Afifi AF, Elnaidany NF. Assessment of Cryptosporidium parvum infection in immunocompetent and immunocompromised mice and its role in triggering intestinal dysplasia. International Journal of Infectious Diseases. 2013; 17:593–600.
2. Akhtar S, Ismail T, Fraternale D & Sestili P. Pomegranate peel and peel extracts: Chemistry and food features. Food Chemistry. 2015; 174: 417–425.
3. Al-Mathal EM and Alsalem AM. Pomegranate (*Punica granatum*) peel is effective in a murine model of experimental Cryptosporidium parvum. Exp Parasitol. 2012; 131: 350–357.
4. Al-Said FA, Opara UL, Al-Yahyai RA. Physico-chemical and textural quality attributes of pomegranate cultivars (Punica granatum L.) grown in the Sultanate of Oman. Journal of Food Engineering. 2009; 90: 129–134.
5. Anderson BC. Moist heat inactivation of Cryptosporidium sp. Am J Public Health. 1985; 75:1433–1434.

6. Armitage, P., Berry, G. and Matthews, J.N.S.: Statistical Methods in Medical Research. Blackwell Science, Oxford (2001).
7. Belal SKM, Abdel-Rahman AH, Mohamed DS, Osman HEH, Hassan NA. Protective effect of Pomegranate fruit juice against Aeromonas hydrophilainduced intestinal histopathological changes in mice. World Appl. Sci. J. 2009; 7: 245–254.
8. Bourlioux P, Koletzko B, Guarner F, Braesco V. The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium ''The Intelligent Intestine'', held in Paris, June 14, 2002. Am. J. Clin. Nutr. 2003; 78: 675–683.
9. Brantley RK, Williams KR, Silva TMJ, Sistrom M, Thielman NM, Ward H, Lima AAM, Guerrant RL. AIDS-associated diarrhea and wasting in Northeast Brazil is associated with subtherapeutic plasma levels of antiretroviral medications and with both bovine and human subtypes of Cryptosporidium parvum. Braz. J. Infect. Dis. 2003; 7: 16–22.
10. Cabada MM, White AC Jr. Treatment of cryptosporidiosis: do we know what we think we know?. Curr Opin Infect Dis. 2010; 23(5):494–9.
11. Certad G, Nqouanesavanh T, Guyot K, Gantois N, Chassat T, Mouray A, Fleurisse L, Pinon A, Cailliez JC, Dei-Cas E, Creusy C. Cryptosporidium parvum, a potential cause of colic adenocarcinoma. Infect. Agent Cancer. 2007; 2, 22.
12. Choi J, Kang O, Lee Y, Che H, Oh C, Brice O, Kim M, Sohn D, Kim H, Park H, Shin D. *In vitro* and *in vivo* antibacterial activity of *Punica granatum* peel ethanol extract against *Salmonella. Evid.* Based Complement. 2009; 690518-1–690518-8.
13. Current WL, Reese NC, Ernst JV, Bailey WS, Heyman MB, Weinstein WM. Human cryptosporidiosis in immunocompetent and immunodeficient persons. Studies of an outbreak and experimental transmission. N Engl J Med. 1983; 308:1252–1257.
14. Dkhil MA. Anti-coccidial, anthelmintic and antioxidant activities of pomegranate (*Punica granatum*) peel extract. Parasitol Res; 2013; 112: 2639–2646.
15. Eisenberg JN, Priest JW, Lammie PJ, Colford JM Jr. The Serologic response to *Cryptosporidium* in HIV-infected persons: implications for epidemiologic research. Emerg Infect Dis. 2001; 7:1004– 1009.
16. Enemark HL, Bille-Hansen V, Lind P, Heegaard PMH, Vigre H, Ahrens P, Thamsborg SM. Pathogenicity of *Cryptosporidium* parvum-evaluation of an animal infection model. Vet. Parasitol. 2003; 113: 35–57.
17. Fawole OA, Makunga NP, Opara UL. Antibacterial, antioxidant and tyrosinase inhibition activities of pomegranate fruit peel methanolic extract. BMC Complementary and Alternative Medicine. 2012; 12:200.
18. Fayer R. Cryptosporidium: a water-borne zoonotic parasite. Veterinary parasitology. 2004; 126(1– 2):37–56.
19. Fischer UA, Carle R, Kammerer DR. Thermal stability of anthocyanins and colourless phenolics in pomegranate (*Punica granatum L.*) juices and model solutions. Food Chemistry. 2013; 138:1800–1809.
20. Fox LM, Saravolatz LD. Nitazoxanide: a new thiazolide antiparasitic agent. Clin. Infectious Dis. 2005; 40:1173-1180.
21. Guitard J, Menotti J, Desveaux A, Alimardani P, Porcher R, Derouin F, Kapel N. Experimental study of the effects of probiotics on *Cryptosporidium parvum* infection in neonatal rats. Parasitol. Res. 2006; 99: 522–527.
22. Hemphill A, Mueller J, Esposito M. Nitazoxanide, a broad spectrum thiazolide anti-infective agent for the treatment of gastrointestinal infections. Expert Opin Pharmacother. 2006; 7:953–964.
23. Henriksen SA, Pohlenz JF. Staining of *Cryptosporidia* by a modified Ziehl–Neelsen technique. Acta Vet Scand. 1981; 22:594–6.
24. Johanningsmeier SD, Harris GK. Pomegranate as a functional food and nutraceutical source. Annu Rev Food Sci Technol. 2011; 2:181-201.
25. Li X, Brasseur P, Agnamey P, Lemeteil D, Favennec L, Ballet JJ, Rossignol JF. Long-lasting anticryptosporidial activity of nitazoxanide in an immunosuppressed rat model. Folia Parasitol. 2003; 50:19-22.
26. Liu H, Shen Y, Yin J, Yuan Z, Jiang Y, Xu Y, Pan W, Hu Y, Cao J. Prevalence and genetic characterization of *Cryptosporidium*, *Enterocytozoon*, *Giardia* and *Cyclospora* in diarrheal outpatients in China. BMC Infectious Diseases. 2014; 14(1):290–292.
27. Manar A. Bashandy, Azza W. Zanaty, Amira M. El-Seidy and Maathir K. El-Shafie. Combined effects of vitamins C and E on acute ethanol toxicity of the liver and jejunum of albino rats: histological, immunohistochemical, and biochemical study. The Egyptian Journal of Histology. 2012; 35:496-508.
28. Maruyama H, Tanaka M, Hashimoto M, Inoue M, Sasahara T. The suppressive effect of Mekabu fucoidan on an attachment of *Cryptosporidium parvum* oocysts to the intestinal

epithelial cells in neonatal mice. Life Sci. 2007; 80:775–781.
29. Moon HW, Schwartz A, Welch MJ, McCann PP, Runnels PL. Experimental fecal transmission of human *Cryptosporidia* to pigs, and attempted treatment with an ornithine decarboxylase inhibitor. Vet Pathol. 1982; 19:700–7.
30. Pedraza-Díaz S, Amar C, Nichols GL, McLauchlin J. Nested polymerase chain reaction for amplification of the *Cryptosporidium* oocyst wall protein gene. Emerg Infect Dis. 2001; 7:49–56.
31. Rehg JE, Hancock ML, Woodmansee DB. Characterization of a dexamethasone treated rat model of *Cryptosporidium* infection. J Infect Dis. 1988; 158:1406–7.
32. Robinson P, Martin P, Garza A, D'Souza M, Mastrangelo MA, Tweardy D. Substance P receptor antagonism for treatment of cryptosporidiosis in immunosuppressed mice. J. Parasitol. 2008; 94: 1150–1154.
33. Rowayshed G, Salama A, Abul-Fadl M, Akila-Hamza S and Emad A. Nutritional and Chemical Evaluation for Pomegranate (Punica granatum L.) Fruit Peel and Seeds Powders By Products. Middle East Journal of Applied Sciences. 2013; 3(4): 169-179.
34. Sasahara T, Maruyama H, Aoki M, Kikuno R, Sekiguchi T, Takahashi A, Satoh Y, Kitasato H, Takayama Y, Inoue M. Apoptosis of intestinal crypt epithelium after *Cryptosporidium parvum* infection. J Infect Chemother. 2003; 9:278–281.
35. Schnyder M, Kohler L, Hemphill A, Deplazes P. Prophylactic and therapeutic efficacy of nitazoxanide against *Cryptosporidium parvum* in experimentally challenged neonatal calves. Vet Parasitol. 2009; 160(1-2):149-54.
36. Singh RP, Chidambara Murthy KN, Jayaprakasha GK. Studies on the antioxidant activity of pomegranate (*Punica granatum*) peel and seed extracts using in vitro models. J Agric Food Chem. 2002; 50(1):81-6.
37. Sparks H, Nair G, Castellanos-Gonzalez A and White AC Jr. Treatment of *Cryptosporidium*: What We Know, Gaps, and the Way Forward. Curr Trop Med Rep. 2015; 2(3):181–7.
38. Suresh P, Rehg JE. Comparative evaluation of several techniques for purification of *Cryptosporidium parvum* oocysts from rat feces. J Clin Microbiol. 1996; 34:38-40.
39. Suvarna SK, Layton C, Bancroft JD. Bancroft's Theory and Practice of Histological Techniques. Elsevier, Churchill Livingstone; 2013.
40. Takeuchi D, Jones VC, Kobayashi M, Suzuki F. Cooperative role of macrophages and neutrophils in host antiprotozoan resistance in mice acutely infected with *Cryptosporidium parvum*. Infect. Immun. 2008; 76: 3657–3663.
41. Viel H, Rocques H, Martin J, Chartier C. Efficacy of nitazoxanide against experimental cryptosporidiosis in goat neonates. Parasitol Res. 2007; 102:163–166. Water samples. Iran J Public Health. 2007; 34:13–18.
42. Vijayanand S, Hemapriya J. In vitro antibacterial efficacy of peel and seed extracts of *Punica granatum L*. against selected bacterial strains. Int J Microbiological Res. 2011;1(4):231–234.
43. Viuda-Martos M, Fernandez-Lopez J, Pérez-Alvarez JA. Pomegranate and its many functional components as related to human health: a review. Comprehensive Reviews in Food Science and Food Safety. 2010; 9:635–654.
44. Warren C, Destura R, Sevilleja J, Barroso L, Carvalho H, Barrett L, O'Brien A, Guerrant R. Detection of epithelial cell injury and quantification of infection in the HCT8 Organoid Model of Cryptosporidiosis. J. Infect. Dis. 2008; 198, 143–149.
45. Watarai S, Tana T, Koiwa M. Feeding activated charcoal from bark containing wood vinegar liquid (nekka-rich) is effective as treatment for cryptosporidiosis in calves. J. Dairy Sci. 2008; 91: 1458–1463.
46. Weyl-Feinstein S, Markovics A, Eitam H, Orlov A, Yishay M, Agmon R, Miron J, Izhaki I, Shabtay A. Short communication: effect of pomegranate-residue supplement on *Cryptosporidium parvum* oocyst shedding in neonatal calves. J Dairy Sci. 2014; 97(9):5800-5.
47. Zierdt WS. Concentration and identification of *Cryptosporidium* sp. by use of a parasite concentrator. J Clin Microbiol. 1984; 20:860–861.

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