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THERAPEUTIC EFFICACY OF A CYSTEINE PROTEASES INHIBITOR (PHENYL VINYL SULFONE) EITHER ALONE OR COMBINED WITH NIGELLA SATIVA ON EXPERIMENTAL CRYPTOSPORIDIOSIS

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Abstract

The present study evaluated the efficacy of a cysteine protease inhibitor (PVS) either alone or combined with *Nigella sativa* in treatment of cryptosporidiosis in immunosuppressed mice. Seventy two mice were divided equally into eight groups: a normal control group (G1). Other received dexamethasone to induce immunosuppression (G2) acted as a dexamethasone immunosuppressed control. Six immunosuppressed groups were inoculated with infective dose of *Cryptosporidum* oocycts: (G3) infected control, (G4 & G5) treated with PVS alone in a dose of 50mg/kg & 100mg/kg respectively, (G6 & G7) treated with same dose of PVS and combined with NS 500mg/kg, and (G8) was treated with Paromomycin 250mg/kg. Oocysts shedding were monitored from 2nd to 24th day post-infection. After mice scarification, blood was assessed for hepatorenal drug toxicity. Sections of ileum were subjected to histopathological examination. Mice treated with PVS 100mg/kg combined with NS showed marked improvement with highest significant efficacy ($P \leq 0.01$) diminished oocyst shedding in G6 & G7 by 91% & 94% respectively**,** versus its efficacy when given alone in G4 & G5 by 83% & 87% respectively or when compared with paromomycin effect in G8 (81%). PVS in different doses showed no toxic effect on hepatorenal parameters.

Key words: Cryptosporidiosis, Cysteine proteases inhibitor, Nigella sativa, experimental study.

Introduction

Cryptosporidiosis was first discovered in the gastric mucosa of mice (Ernest Edward Tyzzer, 1907) and in human (Nime *et al*, 1976). *Cryptosporidum* is an intracellular protozoan family Cryptosporidiidae, phylum Apicomplexa. Two species are blamed in human infections; *C. hominis* that confined to man with anthroponotic transmission and *C. parvum* infecting a wide variety of animals especially livestock with zoonotic transmission (Leitch and He, 2011). *C. parvum* was accounted as a pathogen for the developing world; although now it is ubiquitous and found to be responsible for several waterborne outbreaks worldwide which was a compelling reason to consider it as a bioterrorism agent (Rotz *et al*, 2002). CDC listed *C. parvum* as a category B pathogen in a bioterrorism agent (Moran, 2002) with estimated hazard ratio 2.3 in toddlers (Kotloff *et al*, 2013). In immunocompetent patients infection gave an acute, self-limiting diarrheal of 1-2 week duration, in immune-compromised persons illness was much more severe (Tallant *et al*, 2016) and became life threatening (Tandon and Gupta, 2014). *C.* oocyst characterized by its resistance to conventional water purification techniques and the low infectious dose, it was determined that the 50% infective dose (ID50) of *C. parvum* for cryptosporidiosis seronegative is only 132 oocysts (Dupont *et al*, 1995) and 10 to 83 oocysts for *C. hominis* (Chappell *et al*, 2006). A patient could excrete up to a billion oocysts (Jokipii *et al*, 1986).

Nitazoxanide (FDA approved drug) is the basic therapeutic treatment for immunocompetent infected persons; nevertheless its role in parasitological clearance is doubtful (Amadi *et al*, 2002). Also it is ineffective in HIV patients (Amadi *et al*, 2009; Zulu *et al*, 2005). Also, Paromomycin for immunocom-

pomised cases was not warranted (Hewitt *et al,* 2000). *C. oocyst* targets intestinal epithelium and excyst within lumen released four infective sporozoites by oocyst wall suture that facilitated by protozoan derived molecules i.e. cysteine and serine proteases (Forney *et al,* 1996) an arginine aminopeptidase (Okhuysen *et al,* 1996; Padda *et al,* 2002) ribosomal proteins, heat shock protein (Snelling *et al,* 2007) and secretory phospholipase A_2 (Pollok *et al,* 2003). Therapies against these molecules were under advan-ced studies (Dinler and Ulutas, 2017).

Highlight for protease inhibitors was attained in research as it plays a crucial role in parasitic cell biology mainly protozoan (McKerrow *et al,* 2006). Cysteine protease inhibitor (K11777) is in late pre-clinical testing for submission with the US Federal Drug Administration (FDA) as an anti-chagasic drug (Sajid *et al,* 2011; Engel *et al,* 2010).

Immune system improvement is greatly supportive and with same importance of antimicrobial drug in cryptosporidiosis treatment in AIDS patients (Gargala, 2008). One of the most immune system supportive substances is *Nigella sativa* (NS) that proved several medicinal properties as anti-oxidant (Burits and Bucar, 2000) anti-inflammatory (El Mezayen *et al,* 2006) immune stimulant (Abel-Salam, 2012) anti-tumor (Banerjee *et al,* 2010) analgesic effect (Bashir and Qureshi, 2010) and antiparasitic properties (Baghdadi and Al-Mathal, 2011). NS proved effective in protozoa as *Blastocystis hominis* (El Wakil, 2007), *Toxoplasma gondii* (Mahdy *et al,* 2016), *Giardia* spp. (Bishara and Masoud, 1992), *Trichomonas vaginalis* (Aminou *et al,* 2016), *Plasmodium yoelli nigeriensis* (Okeola *et al,* 2011).

The current study aimed to evaluate the efficacy of a cysteine protease inhibitor (PVS) either alone or combined with *Nigella sativa* (NS) in treatment of cryptosporidiosis in immunosuppressed mice versus to a traditional anticryptosporidial drug, paromomycin®.

Material and Methods

Stool samples collection and examination: Thirty nine stool samples were taken from immunosuppressed patients in dialysis unit and oncology unit Suez Canal University Hospital complaining of diarrhea, from May 2016 to March 2017, after accepting participation with written informed consent. Samples were examined by direct saline and iodine smear, sedimentation procedures, Sheather's sugar flotation technique and Modified Ziehl-Neelsen acid fast stain (Garcia, 2007). *C. oocyst* was detected in ten samples without any other parasitic co-infections. These samples were used for completion of the study

Cryptosporidum purification: Positive stool samples were passed through stainless steel mesh sieve then preserved in 2.5% potassium dichromate, mixed, and stored at 4°, *C. Oocysts* purification was done on discontinuous sucrose gradient as described by Arrowood and Sterling (1987). Briefly, Sheather's sucrose solution was prepared of two dilutions 1:2 & 1:4. Layering 1ml of the 1:4 solutions slowly over layer of 10ml of the 1:2 solution then thirty milliliters of the fecal oocyst suspension was layered onto the 1:4 gradient. The tube was centrifuged at 4,500 for 30min at 4°C. The oocysts-enriched layer was the layer occupying the interface between the sucrose layers. This layer was diluted with 20ml saline and centrifuged at 4,500 for 10min. Oocysts were counted by a hemocytometer then the suspension containing the required concentration for the infection ($1x10^4$ oocysts/ml) was prepared by dilution of the organism in the appropriate amount of distilled water (Gafaar, 2007).

Drugs: Preparation of Phenyl Vinyl Sulphone (PVS) concentrations: Cysteine protease inhibitor, phenyl vinyl sulfone powder (Sigma-Aldrich, Germany) was added to distilled water at concentration of 5mg/ml; it was heated until totally dissolved. The stock solution was kept at 4°C until it was diluted. Treatment began on 5^{th} dpi and continued for 12 days. The doses were 50mg/Kg and

100mg/Kg once daily by oral route (Farid *et al*, 2013).

Nigella sativa: *Nigella sativa* seed oil used was in the form of soft gelatinous capsules supplied by Pharco Pharmaceutical Co. (Alexandria). Each capsule contains 450mg of *N. sativa* seed oil. It was given to the proper mice group with dose of 500mg/kg/day (Farah *et al*, 2004) by stomach tube after its extraction from the capsules from the 5th dpi and continues for 12 days.

Paromomycin (Sigma Aldrich): An aminoglycoside used as anti-cryptosporidiosis drug in immunosuppressed cases; it was dissolved in PBS at 125mg/Kg twice a day (Ndao *et al*, 2013).

Dexamethasone sodium phosphate: (EIPICO, 10th of Ramadan City): A synthetic cortico-steroids used for immunosuppression that was given as oral dose of 0.25µg/g/day for 10 successive days before the inoculation of *C. oocysts*, the same dose was maintained throughout the experiment (Rehg, 1994).

Animal model for experimental infections: Swiss albino mice were purchased from faculty of veterinary medicine Suez Canal University. They were aged 3-5 weeks with average weight between 25 & 35g. Housed under specific-pathogen-free condition in well ventilated cages with perforated covers, Bedding was changed every day. The mice were allowed to acclimatize laboratory environment for one week before the start of the experiment.

The experimental work was conducted in accordance with the internationally accepted principles for laboratory animal use and care as found in the United States guidelines (United States National Institutes for Health publication no. 85-23, revised in 1985). Mice stool was examined by direct wet saline smear, iodine and Sheather's sugar flotation method (Garcia, 2007), to exclude the presence of parasites.

Seventy two mice were divided randomly into eight equal groups (of nine each) as following: G1: Negative control, G2: Immunosuppresed control and G3: Positive infected immunosuppressed control.

G4 to G8 (experimental mice) were infected immunosuppressed and received drugs as following: G4 & G5: received PVS alone in a dose of 50mg/kg &100mg/kg respectively, G6 & G7: received PVS in a dose of 50mg/kg & 100mg/kg respectively that combined with NS 500 mg/kg and G8: received Paromomycin 250 mg/kg.

Proper treatment regimens were given from 5th dpi and continued for 12 days.

Mice scarification: At the 24th dpi, mice were sacrificed under light diethyl ether anaesthesia. From each mouse, ileum was rapidly removed and fixed in 10% formol saline and stained with H&E for histopathological study (Bancroft *et al*, 1996). Blood was taken using insulin needle 29 gauge by heart puncher. Then it was left to clot at 4C^{0} after that centrifugation for 10 min at 3000 rpm. Delicate aspiration for separated serum from clotted blood was done for assessment of drug toxicity.

Parameters for drug efficacy evaluation: a-Monitoring clinical signs and survival rate in each group. b-The oocytes' shedding was done daily from the 2nd to the 24th dpi for each mice group. Fresh fecal pellets were collected every day from each mouse in the studied groups over the experimental period, each sample was suspended in 10% formalin and homogenized, then, 1 mg was prepared as a fecal smear and stained by modified Ziehl-Neelsen staining method. Oocytes' counts per 50 fields at magnification ×100 were scored. Calculation of the inhibition rate % (x) was done to evaluate oocysts shedding reduction in (G4, G5, G6, G7 & G8) compared to G3, it was done according to the following equation:

$$X = \frac{\text{Oocyst per gram of feces in + ve control group} - \text{Oocyst per gram of feces in treated group}}{\text{Oocyst per gram of feces in + ve control group}} X\ 100$$

The histopathological features of mice intestinal sections were examined. Assessment of drug toxicity: Measuring liver function; serum enzymes alanine transaminase (ALT) and aspartate transaminase (AST) and measuring kidney function; serum creatinine and serum urea levels. Measurements were done by using a clinical automatic analyzer (Hitachi, Japan) and commercial reagent kit (Roche Diagnostic, Mannheim, Germany) according to the manufacturer's protocol.

Statistical analysis: Oocysts shedding were performed on different days, means ± S.D. Efficacy and inhibition rates (%) were calculated. Significance of inhibition was tested using a paired Student's *t*-test. For drug toxicity assessment statistical analyses were performed using one-way ANOVA, and means were compared using Duncan's multiple range tests as a post hoc test at the 5% probability level. P value < 0.05 was regarded significant.

Results

Clinical signs and survival rate: Monitoring daily activity and body weight of mice revealed average diminution for both of them in all infected groups (G3-G8), which regained only in treated groups with variable degrees, while infected untreated group appeared to be sick by wrinkled skin and hunched posture furthermore their feces became softer. Survival rate was 66.7% in G3 (three mice were died). While in G4 and G8 it was 77.8 %, two mice were died in each of them. Otherwise the remaining mice were kept alive till the study end.

Oocysts shedding and reduction % in experimental groups: Reduction in the median number of oocysts in treated versus infected control mice were calculated (Fig. 1 & Fig. 2). It was revealed that infected non-treated control mice (G3) shed more oocysts than all other groups with gradual progressive increase from 8^{th} dpi with peak at 12^{th} day. It was gradually diminished till 14^{th} day when it showed another ascending increase at 16^{th} dpi then gradual decrease. PVS treated mice G4 & G5 showed lower oocyst shedding than non-treated group G3 which was more significant in G5 especially from 16^{th} dpi till the study end (Fig. 1). Mice treated with PVS combined with NS showed much more significant decrease in oocysts shedding. In G7 there was great decreased to minimal amount at 12^{th} dpi till the study end (Fig. 2). *Cryptosporidium* oocysts isolated from human feces were given (Fig. 3).

Histopathological changes: Examination of ileum of G1 showed normal histological architecture (Fig. 4A). Examination of intestine from infected mice revealed presence of *C. oocysts* of different developmental stages near the intestinal epithelium brush border (Fig. 4B). There were necrosis of the intestinal epithelium, sloughing of the villi, edema and infiltration of inflammatory cells in the lamina propria (Fig. 4C). In some cases, there were thickening and flattening of the villi with hyperplasia of the intestinal epithelium (Fig. 4D). In PVS treated group, the villi were short and thick. There was infiltration of inflammatory cells in lamina propria and sub-epithelial edema (Fig. 5A). Mice treated with PVS combined with NS, the ileum showed few inflammatory cells and mild edema in lamina propria. Intestinal epithelium was slightly normal (Fig. 5B). Ileum of Paromomycin treated mice showed blunting of villi, sub-mucosal edema and hyperplasia of goblet cells (Fig. 5C).

Assessment of drug toxicity:

Table 1: Clinical biochemistry parameters in different mice groups

groups	Liver function test		Kidney function test	
	ALT U/L	AST U/L	Serum creatinine mg/dl	Serum urea mg/dl
G1	48.7 ± 7.56 [a]	67.1 ± 5.83[a]	0.53±0.04[a]	50.86±8.83 [a]
G2	92.8 ± 5.52[b]	112.1 ± 9.21[b]	0.52±0.06 [a]	52.91±6.03 [a]
G3	116.4 ± 5.76[c]	139.3 ± 6.74[c]	0.55±0.03 [a]	56.11±5.34 [a]
G4	98.9 ± 4.32[b]	110.6 ± 12.63[b]	0.57±0.04 [a]	54.50±8.20 [a]
G5	88.7 ± 6.50[b]	106.4 ± 8.46 [b]	0.50±0.07 [a]	53.12±7.51 [a]
G6	53.8 ± 4.62[a]	78.5 ± 4.73 [a]	0.54±0.05 [a]	51.08±6.42 [a]
G7	50.1 ± 7.86[a]	75.7 ± 5.38 [a]	0.52±0.04 [a]	51.28±3.53 [a]
G8	61.1 ± 6.32[a]	78.3 ± 6.25 [a]	0.54±0.02 [a]	55.19±2.73 [a]

One way ANOVA-test followed by Duncan's Multiple Range Test; n=9 ($P \leq 0.05$) Means with different alphabetical super-scripts in the same column are significantly different (at $P \leq 0.05$) and vice versa.

Discussion

The present work examine the efficacy of in vivo activity of a cysteine protease inhibitor (PVS) either alone or combined with NS for eradication of cryptosporidiosis in immunosuppressed mice. Dexamethasone was used for mice immunosuppression that caused increasing susceptibility to cryptosporidium infection, through immune system suppression, by decreasing of B cell count in the spleen and changing CD4 + /CD8 + ratio (Üner *et al,* 2003). Dexamethazone also lead to short incubation period and prolonged oocyte shedding this was in accordance with (Matsui, 2001) who found oocyst shedding in immunosuppressed mice to be about 24 days, and Lacroix, (2001) who noticed it to be 3-4 weeks. Monitoring clinical signs and survival rate in each group revealed that survival rate in immunosuppressed infected non treated group was 66.7% which agreed with Tarazona *et al.* (1998) who reported 66% survival rate in post cryptosporidial infected animals. Clinical signs were ameliorated in all treated groups compared to non-treated groups, gradual weight and activity regaining was obtained only in treated groups. The present work showed that protease inhibitor (PVS) has a therapeutic effect in a dose dependent manner; it was more effective in 100mg\Kg than 50 mg\kg, either it was given alone or combined with NS.

A panel of VS related compounds was proven to give marked inhibition of cysteine peptidases from many protozoa (Ang *et al,* 2011; Sajid *et al,* 2011; Abdulla *et al,* 2008; Steverding *et al,* 2006) and helminthes (Helmy *et al,* 2008; Abdulla *et al,* 2007). Okhuysen *et al*. (1996) reported that overwhelming infection in the cryptosporidiosis immunosuppressed patients is owed to auto-infection by thin walled oocyst rather than thick walled one, so challenging thin walled oocyst by excystation antagonist may have a successful effect.

Researches attributed success of protease inhibitor in treating parasite to two main factors, firstly: inhibition selectivity by differential uptake of parasite proteases rather than its human host homologues, Secondly: lesser amount of protease gene families in parasites, so host-origin lysosomal cysteine proteases are much higher than that for the parasite proteases, thus host protease activity is difficult to be eliminated in a short course treatment targeting an acute infection (McKerrow *et al*, 1999).

Both two lines of treatment (PVS alone or combined with NS) have succeeded to alter oocyte shedding pattern to be better than the untreated group and to be more effective than paromomycin. It was gradually decreesed after PVS alone treatment in G5 more than in G4 which was apparent on 16^{th} dpi and the reduction on 12^{th}, 14^{th} 16^{th} & 18^{th} dpi was significant $P \leq 0.05$ efficacy of treatment were 87% and 83% in G5 & G4 respectively (Fig. 1) On the other hand highly significant differences was attained in PVS treated groups combined with NS specially in G7 at 12^{th} dpi which was earlier than that reached by PVS alone, the efficacy rate reached to highest level (94%) in G7 and (91%) in G6 that was highly statistically significant ($P \leq 0.01$). Oocyst shedding gradually decreased to very low levels by

24^{th} dpi. So, PVS at a dose of 100mg\Kg combined with NS is the superior choice in treating cryptosporidiosis immunosuppressed mice.

Regarding NS it helped in amelioration of infection and decreased oocyst shedding; this was in accordance with (Elrefaii, 2003). This was possibly through direct immune reconstitution effect, its antioxidants properties and its active biocide ingredients like alkaloid nigellicine and thymoquinone (Darakhshan *et al*, 2015). Active biocide ingredients could limit growth and development of protozoan parasite (Seddiek and Metwally, 2013).

High efficacy of the combination (PVS & NS) in challenging cryptosporidiosis was explained by potential effect of PVS that directly interfere with oocyst excystation and host cell invasion; with the synergism of immune reconstitution of NS. Combined effect of drug therapy and immune system strengthen in treating cryptosporidiosis was proved, in a study that used nitazoxanide and highly active anti-retroviral therapy (HAART) in treatment of HIV cryptosporidiosis infected patients resulting in reduction of prevalence of infection; that was explained by combined direct effect of aspartyl protease inhibitor included in HAART and reconstitutional immune effect (Zardi *et al*, 2005). Immune status amelioration has a critical function in cryptosporidiosis clearance, especially in immune-compromised hosts; aggregated intraepithelial lymphocytes and $CD4^+$ T cells gave higher reduction of *C. oocyst* load in infected mice (Tessema *et al*, 2009). Cryptosporidiosis clearance post anti-retroviral therapy was relapsed if CD4+ counts declined (Maggi *et al*, 2000).

Histopathological changes in G3 (control positive group) was explained with that parasites forms invaginate itself in microvillus border causing loss of the mature surface epithelium. This leads to marked shortening and fusion of the villi and lengthening of the crypts that is tried to be compensated by accelerated cell division. The parasite may enhance apoptosis in adjacent epithelial cells while inhibiting apoptosis in the infected cells to facilitate prolonged survival of the parasites (O'Hara and Chen, 2011).

Several vital physiological functions are carried out in the liver, amongst of them is detoxification. ALT and AST are biomarker enzymes that reflect functional integrity of liver. In the present study both liver enzymes were markedly elevated in G2 and G3 with significant result ($P<0.05$) compared to G1. These may attributed to corticosteroid therapy, while regressions of enzymes level occurred in the other infected treated groups with variable degrees. This result agreed with Hagopian *et al.* (2003) who found that enzymes including ALT were produced by intermediary metabolism occurring due to gluconeogenic and glycol-genic properties of dexamethasone. Hassona *et al.* (2017) reported a significant elevation in AST & ALT activities after dexamethasone administration due to the liver damaged structural integrity. Regression of these enzymes in other infected treated groups may explained in PVS group on the basis of riding off the infection helped partially in regaining liver function while in G6 & G7 with combined PVS & NS therapy, the main cause of serum enzyme regression was that the effect of NS, which may be induced partly by improving the immunological host system and to some extent with its antioxidant effect (Mollazadeh and Hosseinzadeh, 2014). Aboelwafa and Yousef (2015) found that supplementation of hydrocortisone-treated rats with thymol reversed most of the biochemical, histological, and ultra-structural alterations of the liver. So, thymol has strong ameliorative effect against hydrocortisone-induced oxidative stress injury in hepatic tissues. Serum creatinine and urea may not affected by dexamethasone or cysteine protease therapy. In groups receiving combined PVS & NS, protective effect of NS was expected. Abdel-Daim and Ghazy (2015) found that NSO reduced serum hepatic and renal injury mark-

ers. It reduced the lipid peroxidation in hepatic and renal tissues and elevated liver and renal antioxidant enzymes and glutathione levels.

Conclusion

The cysteine protease inhibitor PVS combined with *Nigella sativa* NS have a great efficacy in improving immune compromised cryptosporidiosis and alleviate the symptoms.

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Explanation of figures

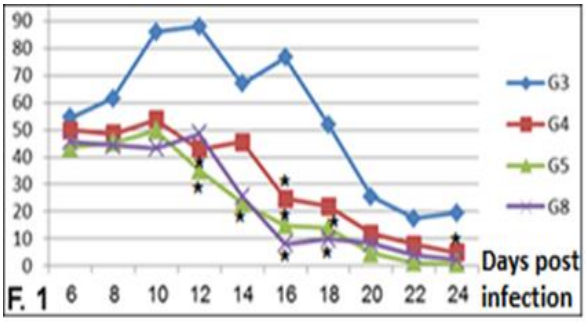
Fig. 1: Efficacy of PVS (G4& G5) and Paromomycin (G8) therapy on oocysts shedding in immunosuppressed infected mice compared to infected non treated mice (G3). *P < 0.05.

Fig. 2: Efficacy of PVS combined with NS (G6& G7) and Paromomycin (G8) therapy on oocysts shedding in immunosuppressed infected micecompared to infected non treated mice (G3). *P < 0.05, **P< 0.01.

Fig. 3: *Cryptosporidum oocysts* in human isolates stained with modified Ziehl–Neelsen acid fast stain (X1000).

Fig. 4: Ileum from control group and cryptosporidium infected group. (A) normal architecture in normal control. (B-D) *Cryptosporidium* infected. (B) *Cyptosporidium oocyst* of different developmental stages in intestinal lumen (arrow). (C) Necrosis of intestinal epithelium, sloughing of intestinal villi, inflammatory cellular infiltration (arrow). (D) Hyperplasia of intestinal epithelium (arrow) H&E.

Fig. 5: Photomicrograph of ileum from different groups (A) PVS treated. Shortage and thickening of villi: Infiltration of inflammatory cells in lamina propria and sub-epithelial edema (arrow). (B) PVS+NS treated group: Few inflammatory cells and mild edema in lamina propria. Intestinal epithelium is slightly normal. (C) Paromomycin treated group. Submucosal edema (star) & hyperplasia of goblet cellsH&E.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Days post infection
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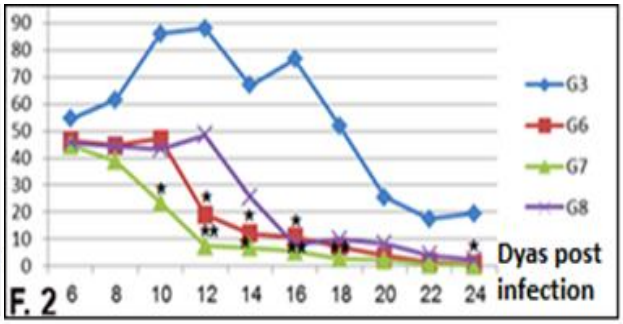
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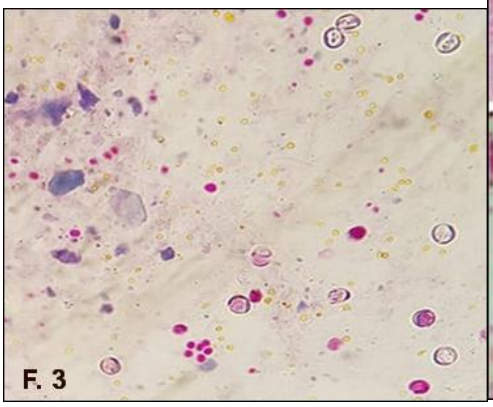
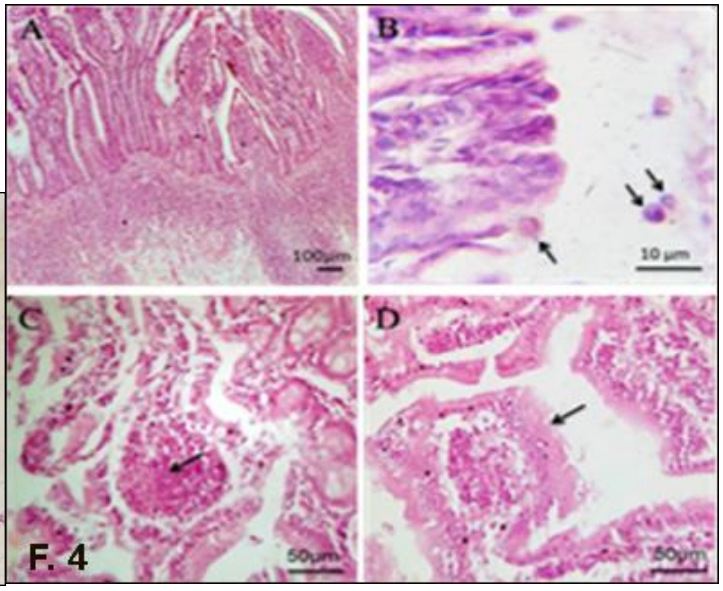

F. 3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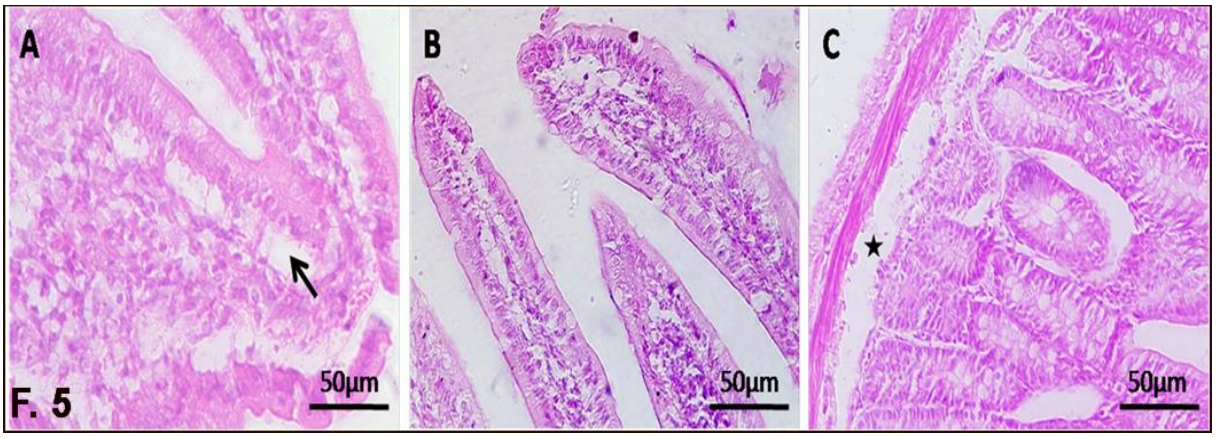
F. 4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