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CHENOPODIUM AMBROSIOIDES OIL EXTRACT REDUCED CRYPTOSPORIDIUM PARVUM DEVELOPMENT IN VIVO

By
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Abstract

Cryptosporidium is a widespread parasite transmitted through contaminated food and water; it could lead to severe gastrointestinal disease in immunocompromised hosts. To date, no specific therapy proved to be effective against *Cryptosporidium parvum* (*C. parvum*). The present work studied the efficacy of Chenopodium *ambrosioides (CA) oil extract on C. parvum infected dexamethasone (DEX*) immunosuppressed mice. Infected immunosuppressed mice (n=70) were divided into 14 groups of five mice each according to different concentrations of received treatments either CA oil extract at doses of 75, 100, 125, 150 & 175mg/kg/day alone or in combination with nanoparticles or nitazoxanide at doses of 50 & 100mg/kg/day alone or in combination with nanoparticles. Five infected non-treated mice were included as an infected control group; five non- infected & non-treated mice were enrolled as a normal control one. Anti-*Cryptosporidium* efficacies of different treatment regimens were evaluated in the 16th mice groups microscopically by detection of *C. parvum* oocysts in fecal pellets and histopathological examination of their small intestines.

The results showed that different concentrations of CA oil extract induced a significant reduction in the mean number of *C. parvum* oocysts. The rate of oocysts reduction was parallel to the increasing concentrations of the drug. The inhibitory effects of CA oil extract on the parasite were enforced by adding nanoparticles. Histopathological examination of mice intestine revealed that CA did not show toxic effect on intestinal mucosa even with high doses.

Keywords: Chenopodium ambrosioides, Nitazoxanide, *C. parvum*, Nanoparticles.

Introduction

Cryptosporidium is an intracellular protozoan parasite infecting different hosts including humans and animals leading to asymptomatic or mild-to-severe gastrointestinal ailments (Ryan *et al,* 2014). About 31 *Cryptosporidium* species have been identified; the most common in humans is *Cryptosporidium parvum* (*C. parvum)* and *Cryptosporidium hominis* (Kvac *et al,* 2016). Cryptosporidiosis is usually a self-limiting disease in immunocompetent hosts, presented in human by abdominal pain, fever, vomiting, malabsorption, and diarrhea. Diarrhea could be sometimes profuse and prolonged or even intractable and fetal in immuno-compromised patients (Bouzid *et al,* 2013).

Nitazoxanide (NTZ) is the proved drug for the treatment of diarrhea caused by *Cryptosporidium* infection (Mainali *et al,* 2013). But, it showed imperfect efficacy in the most vulnerable patients (Manjunatha *et al,* 2016). *Chenopodium ambrosioides* (CA) is a herbal plant of family Chenopodiaceae, widely distributed all over the world, as one of the commonest effective medicinal plants (Oliveira-Tintino *et al,* 2018). CA has wide biological actions exerted by active ingredients and secondary metabolites of its extracts or oils (Barros *et al,* 2013). The oil had 72.7% α-terpinene (commonest constituent), 15% p-cymene and 7.2% ascaridole (Baldissera *et al,* 2016). Its leaves act as an anti-helminthic and vermicide (Alitonou *et al,*

2012). It also used in the management of disease of different systems (gastrointestinal, respiratory, vascular, and nervous diseases) and to treat diabetes and dyslipidemia. It had sedative, antipyretic, and anti-rheumatic effects (De genhardt *et al,* 2016).

Nanotechnology has the potential for restoring the use of toxic drugs through the use of complex structures that allow carrying drugs being able to achieve only the pathogen, preserving the host cells and exerting its effect with less toxicity, improved selectivity consequently greater efficacy (Forrest and Kwon, 2008).

In parasitic diseases, nanotechnology was employed as drug delivery systems such as in toxoplasmosis, leishmaniasis, malaria and trypanosomiasis (Date *et al,* 2007). Nanotechnology represents a useful drug delivery system to improve the pharmacokinetic profile of drugs. The bioavailability increased with the enhancement of solubility, stability, dissolution rate, and surface area, and modulating the therapeutic action and permeability of the drug through the absorptive membranes, leading to lower drug doses use (Joshi *et al,* 2004). As there was a clear need for effective anti cryptosporidial drugs (Ryan *et al,* 2016).

This study aimed to evaluate the anti-*Cryptosporidium* therapeutic effect of CA oil extract alone or in combination with nanoparticles as compared with nitazoxanide.

Materials and Methods

Ethical considerations: The study was conducted at Theodor Bilharz Research Institute (SBSP/TBRI, Giza, Egypt) in strict accordance with the TBRI Guidelines for Ethical Conduct in Use of Animals in Research.

Experimental animal: Albino C57 BL/6 male mice (n=80) aged 6-8 weeks and weighing 16-19g, were used. Mice were maintained in conditioned rooms at 21°C on sterile water and a balanced dry food containing 14% protein.

Experimental protocol: *C. parvum*- infected mice (n=75) which were previously immunosuppressed with synthetic corticosteroids Dexamethasone (DEX) were enrolled in the study and were equally randomized to 14 groups of five mice each, according to different types and doses of received treatments; GI: infected treated with 75mg/kg of CA, GII, infected treated with 75mg/kg of CA + nanoparticles; GIII, infected treated with 100 mg/ kg of CA; GIV, infected treated with 100 mg/kg of CA + nanoparticles; GV, infected treated with 125mg/ kg of CA; GVI, infected treated with 125mg/kg of CA + nanoparticles; GVII, infected treated with 150mg/kg of CA; GVIII, infected treated with 150mg/kg of CA+ nanoparticles; GIX, infected treated with 175mg/kg of CA; GX, infected treated with 175mg/kg of CA+ nanoparticles; GXI, infected treated with 50mg /kg of nitazoxanide; GXII, infected treated with 50mg/kg of nitazoxanide + nanoparticles; GXIII, infected treated with 100 mg/kg of nitazoxanide (the standard dose); GXIV, infected treated with 100 mg/kg of nitazoxanide + nanoparticles. Besides, five infected non-treated mice were included as infected control group (GXV), and five non-infected non-treated mice were enrolled as the normal control group (GXVI).

Immunosuppression was induced by given mice DEX orally at a dose of 0.25mg/ day for 14 successive days before infection with *C. parvum* oocysts (Rehg *et al,* 1988).

Parasites and infection: Purified *C. parvum* oocysts in phosphate-buffered saline (PBS) pH7.2 were obtained from Theodor Bilharz Research Institute (SBSP/TBRI, Giza) and stored at 4^{o}C. Oocysts were surface sterilized by suspension in 10% (vol/vol) commercial bleach solution (sodium hypochlorite), washed three times in PBS, and enumerated in the Neubauer hemocytometer. Mice were infected by oral gavage, using $1x10^{6}$ *C. parvum* oocysts (Abdou *et al,* 2013).

All mice groups except normal control ones were infected on the 15^{th} day with DEX administration to immunosuppressed groups (Reese *et al,* 1982; Moon *et al,* 1982). Two weeks post-treatment, all mice were sacrificed. Stool samples were examined microscopically for *C. parvum* oocysts and ileal specimens were examined histopathologically for drug toxicity (Gaafar, 2007).

Chenopodium ambrosioides (CA) oil extract: The oil extract was prepared in the Department of Pharmacognosy, Faculty of Pharmacy, Al-Azhar University. Efficacy of the oil was evaluated using a series of upgraded concentrations 100, 200, 300 & 400 parts per million (ppm) each for 3 to 48hrs exposure time according to the experimental design. Each experimental concentration was set in triplicate, and all experiments were conducted at room temperature (28ºC). Control group with drug solvent was run with each experiment (Salama *et al,* 2012), 100 mg of the oil was dissolved in 1ml Tween 80. A total of 99ml of distilled water was added. Solution contained 1/1000 concentrate. This stock solution was used to prepare the required concentrations by add distilled water. After 3 days from infection establishment by shedding of oocysts in stool, the drug was introduced orally for 3 successive days.

Nitazoxanide (NTZ) is manufactured by Al Andalous Medical Co. Cairo, as a suspension. The drug was administrated orally in a daily dose of 50 &100mg/kg body weight started 3rd-day post-infection for 3 successive days (Theodos *et al,* 1998).

Preparation of solid nanoparticles: Synthesis procedure was carried out followed the ionotropic gelation technique (Ohya *et al,* 1994). Chitosan (degree of deacetylation of 93%) was manufactured and provided by (Sigma-Aldrich, USA), Sodium Tripolyphosphate (TPP), Phosphate buffer saline, and Acetic acid were commercially available and of analytical grade. Approximately 2% of the chitosan was dissolved in a 1.0% (v/v) acetic acid solution. A sodium TPP solution was also prepared in distilled water at a concentration of 5mg/ml. The sodium TPP solution was added drop-wise using a burette to the chitosan solution while stirring (Drop-wise 5ml of the chitosan solution was added to 2 ml of TPP solution under magnetic stirring; 1000 rpm for 1hr) at room temperature followed by sonication for 20min. Nanoparticles were separated by centrifugation at 15,000 rpm at 14°C for 30 minutes. Freeze-dried nanoparticles were stored at 5±3°C. The freeze-dried nanoparticles weights were measured.

CA and NTZ loading chitosan-based nanoparticles (CSNPs): CA oil extract and NTZ-loaded CSNPs were formed spontaneously up-on drop-wise addition of an aqueous solution of sodium TPP to 20ml of a 0.35% w/v chitosan solution, containing 40 mg/Kg CA or NTZ with constant stirring, followed by sonication. The resulted nanocomposite was collected by centrifugation (4 times at 11,000 rpm), washed with distilled water, and dried CA and NTZ-loaded nanoparticles were separated from the aqueous suspension by centrifugation at 20,000g & 14°C for 30 minutes. CA and NTZ release from the nanocomposite was done at room temperature using a 0.01mol/L phosphate-buffered saline (PBS) solution, at pH 7.4, at 215nm. Approximately 85mg of the nanocomposite was added to 500ml of PBS. The amount of CA and NTZ- released into the solution was measured at preset time intervals using a Shimadzu UV-1601 spectrophotometer.

CA & NTZ- loading and encapsulation efficiencies: Encapsulation and loading efficiencies of the nanoparticles were determined by first separating the nanoparticles from the aqueous medium by ultracentrifugation at 15,000 rpm for 30min. The amount of free CA and NTZ-in supernatant was measured by using a UV spectrophotometer at 215nm. CA & NTZ - loading and encapsulation efficiencies of nanoparticles were calculated as follows: Encapsulation efficiency% = $(T_p-T_f/T_p) \times 100$ (1), Loading efficiency% = $(T_p-T_f/\text{mass of nanoparticles}) \times 100$ (2), where T_p = total CA or NTZ amount, T_f = free CA or NTZ amount in supernatant.

Determination of oocyst shedding in infected mice: Fecal pellets were collected from mice to monitor oocyst shedding throughout the experiment. Pellets were re-suspended in a volume of 2.5% potassium dichromate approximately equal to twice that of the feces

and stored at 4°C. Fecal suspensions were smeared onto slides and examined microscopically for the stained oocysts by acid-fast (Ziehl-Neelsen) stain. Number of oocysts in 10 fields under the x100 oil immersion objective lens was calculated (Henriksen and Pohlen, 1981; Abdou *et al,* 2013).

Scarification: Scarification was done two weeks post-treatment by rapid decapitation of all mice. ileal sections were dissected from individual mice, fixed in 10% formalin and subjected to histopathological studies.

Histopathological examination: The last 2 cm of the sacrificed mice ileum was submitted for routine histopathological processing at the Department of Pathology, TBRI, where they were fixed in 10% neutral buffered formalin, dehydrated in ascending grades of ethanol, followed by immersion in xylene, and then impregnated in paraffin. One 5-mm thick section was taken from each block and stained with hematoxylin and eosin (H&E), and then examined by light microscopy to assess histopathological changes (Tzipori *et al,* 1981). Mucosal changes such as crypt villous ratio, blunting of villi, degree of atrophy, ulceration, dysplasia, and lamina propria changes such as degree of inflammatory infiltration, the status of blood vessels, and edema were recorded.

Statistical analysis: Data were collected, tabulated, and statistically analyzed by using statistical package for Social Sciences Program version 18 (SPSS Inc. Chicago, Illinois, USA). Descriptive statistics included mean, SD, and percentage. Analytical statistics included Fisher's exact test, Student's t-test, & Mann-Whitney test. Significance was considered when a P value less than 0.05.

Results

Oral administration of different concentrations of CA oil extract for 3 successive days induced a significant reduction in the mean number of *C. parvum* oocysts. The reduction was parallel to increasing drug concentrations. *C. parvum* oocysts numbers were reduced (11.48%) in infected mice received 75mg/kg compared to control. Reduction rate (Fig. 1) increased by doses 100mg/ kg (19.01%), 125mg/kg (47.94%), 150mg/kg (84.33%) and 175mg/ kg (96.02%)

Inhibitory effect of CA oil extract on the development of *C. parvum* was enforced by nanoparticles addition. The reduction with different doses was: 26.3%, 50.4%, 65.18%, 96.02% & 98.97% for doses 75, 100, 125, 150 & 175mg/kg with nanoparticles respectively (Fig. 2).

The NTZ 50mg/kg showed a reduction of 54.73%, elevated to 78.17% with nanoparticles, but 100mg/kg dose produced a reduction of 63.81%, elevated to 82.25% by adding nanoparticles (Fig. 3).

Histopathological examination of ileal sections in infected non-treated mice showed changes in the morphology of ileal mucosa and lamina propria in the form of mild ulceration, blunting and shortening of villi, villous atrophy, and moderate non-specific inflammatory exudate mainly lymphocytes at lamina propria and villi. The infected mice and treated with175 mg/kg of CA oil extract showed marked improvement in the form of returning of the villous like pattern, villous-crypt ratio. Different histopathological parameters were assessed in H& E-stained slides as given in (Figs. 4A, B, C, D, E, & F).

Discussion

Cryptosporidiosis is a prevalent disease worldwide. Malnourished and immunocompromised individuals are more susceptible to infection. Food-borne and water-borne outbreaks of cryptosporidiosis were reported in different countries (Checkley *et al,* 2015; Abouel-Nour *et al,* 2016). Many antimicrobial drugs have been tested in animals or humans infected with Cryptosporidium sp., but none has been consistently effective against this parasite. There is a clear requirement, therefore, to intensify the search for anti-cryptosporidial drugs.

People in ancient times use medicinal plants without knowledge about active ingredients. In the modern era, curative effects and active ingredients of medicinal plants were elucidated by different scientific techniques (Kaur *et al,* 2018). The parasites developed resistance to different anti-parasitic drugs.

Thus, different health facilities recommended the usage of medicinal plant extracts due to its efficacy and safety (Al-Snafi, 2016). Oil of CA has been used long ago worldwide to treat parasitosis in animals and humans (Singh *et al,* 2011; Eguale and Giday, 2009).

In the current study, the onset of oocyst shedding in stool was on the second day PI in immunosuppressed mice. This agreed with El Shafei *et al.* (2017) who found that the onset of oocyst shedding in stool was on the second day PI in immunosuppressed mice and the fourth day in the immunocompetent groups, while Chai *et al.* (1999) found that oocyst shedding started on fourth day PI in both immunocompetent and immunosuppressed rats.

In the present study, CA oil extract was more effective than NTZ and reduced parasite development by more than 90% at the highest concentrations used (150 & 175mg/kg). Another study reported that administration of upgrade doses of CA oil extract for 3-5 successive days' led to a significant decrease in the mean number of *C. parvum* oocysts in experimentally infected mice and chicks and complete disappearance of oocysts was achieved after administration of 160mg/kg for 3 successive days without any side effect (Khalifa *et al.* (2017). Also, previous studies demonstrated that Nitazoxanide improved diarrhea and reduced mortality rates among *C. parvum* infected, malnourished children. But, the response rate in malnourished children was only 56% (Amadi *et al,* 2002; Rossignol *et al,* 2006). Amadi *et al.* (2009) reported the little effect of NTZ therapeutic efficacy; even with high dose prolonged treatment in immunosuppressed HIV infected children. Similar results were reported by Abubakar *et al.* (2007) and Manjunatha *et al.* (2016) who found inadequacy of NTZ in presence of immunocompromisation status.

In the present work, nanoparticles were valuable as it augments the action of CA and NTZ in a dose-dependent manner. The present study used nanoparticles as recommended by Cameron *et al.* (2016) who found that high concentrations of nanoparticles led to *Cryptosporidium* oocysts destruction, and lower concentrations also affected viability. They added that sporozoites were more sensitive to nanoparticles and destructed with the oocyst membrane leading to oocyst disruption. Similar observations have been reported by other authors (Elmi *et al*, 2013; Su *et al*, 2014; Saad *et al*, 2015).

No doubt, the small intestine morphology is considered as the main indicator of normal gut histology and defined by villus height and crypt depth (Laudadio *et al,* 2012).

In the present study, marked histopathological changes were observed in the infected control mice group. This agreed with Soufy *et al.* (2017) who found that *Cryptosporidium* spp. displaced brush borders resulting in shortening and fusing of the villi and that villous atrophy was explained by toxins secreted by *Cryptosporidium* spp. that directly damage the epithelial cells.

In the present histopathological study, there were marked anti-inflammatory effects of CA oil extracts. This agreed with Jump and Levine (2004) who suggested the possible anti-inflammatory actions of CA extract on intestine by gene expression and histological assays. Also, studies demonstrated that the administration of reasonable doses of CA extracts has an anti-inflammatory effect on mammals through down-regulation of inflammatory genes (Chen *et al*, 2013; Trivellato-Grassi *et al,* 2013; Pomari *et al.* 2014).

Conclusion

The present data proved the efficacy and safety of CA oil extract in reducing *C. parvum* development in vivo. Nanoparticles augment the therapeutic effect of CA and NTZ without affection of safety profile.

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Explanation of figures

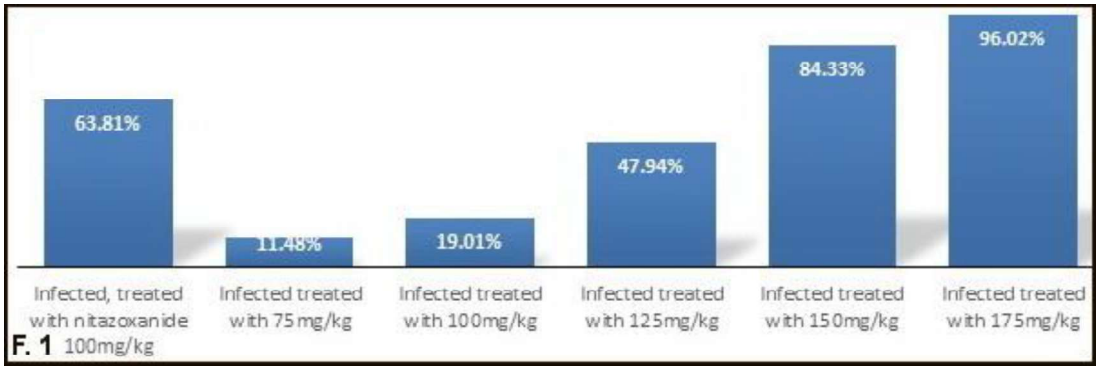
Fig. 1: Anticryptosporidial activities of different concentrations of CA oil extract compared to standard dose of NTZ.

Fig. 2: Nano particles increased anticryptosporidial activities of NTZ and CA oil extract.

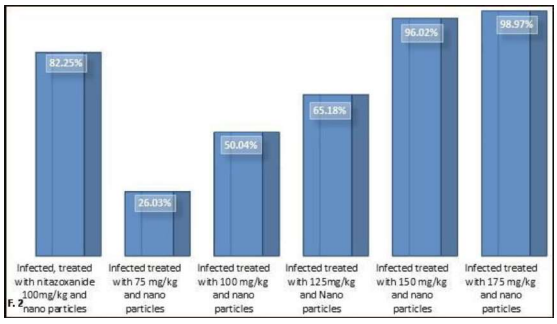
Fig. 3: Nano particles reduced NTZ dose of significant anticryptosporidial activity.

Fig. 4: Histopathological sections of small intestine showed (A) Normal control with normal histological mucosal structures as villi (black arrow), crypt of Lieberkühn (red arrow) and muscular layers (yellow arrow), and normal small intestinal crypt villous ratio. (B) Infected control showed mild ulceration, blunting and shortening of mucosal villi and villous atrophy (red arrows), moderate non-specific inflammatory exudate of lamina propria with lymphocytes (yellow arrow) and villi (green arrow), numerous number of *Cryptosporidium* oocysts adhere on epithelial cell (red circle). (C) Iinfected animal treated with 100mg/kg of CA showed blunting and shortening of mucosal villi in and villous atrophy (red arrows), mild nonspecific inflammatory exudate with lymphocytes in villi (black arrow), moderate oedema in lamina pro-

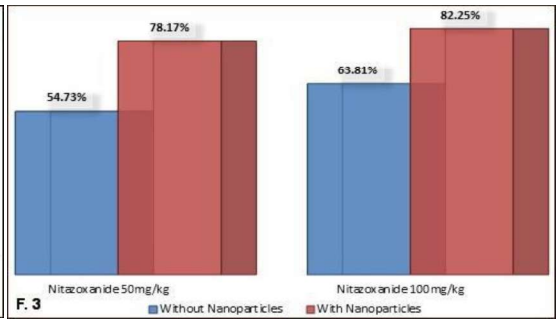
pria, mild number of *Cryptosporidium* oocysts adhere on epithelial cell (black circle). (D) Infected animal treated with CA 125mg/kg body weight/day showed normal histological mucosal structures as villi (black arrow), crypt of Lieberkühn and muscular layers (yellow arrow), mild oedema in lamina propria, few *Cryptosporidium* oocysts adhere to epithelial cell (red circle).(E) Infected animal treated with CA150mg/kg body weight/day showed normal histological mucosal structures as villi (black arrow), crypt of Lieberkühn (red arrow) & muscular layers (yellow arrow), mild oedema in lamina propria, scattered *Cryptosporidium* oocysts adhere on epithelial cell (red circle).(F) Infected mice treated with CA175mg/kg body weight/day showed normal histological mucosal structures as villi (black arrow), crypt of Lieberkühn (red arrow) and muscular layers (yellow arrow), (H&E, x100, x200).



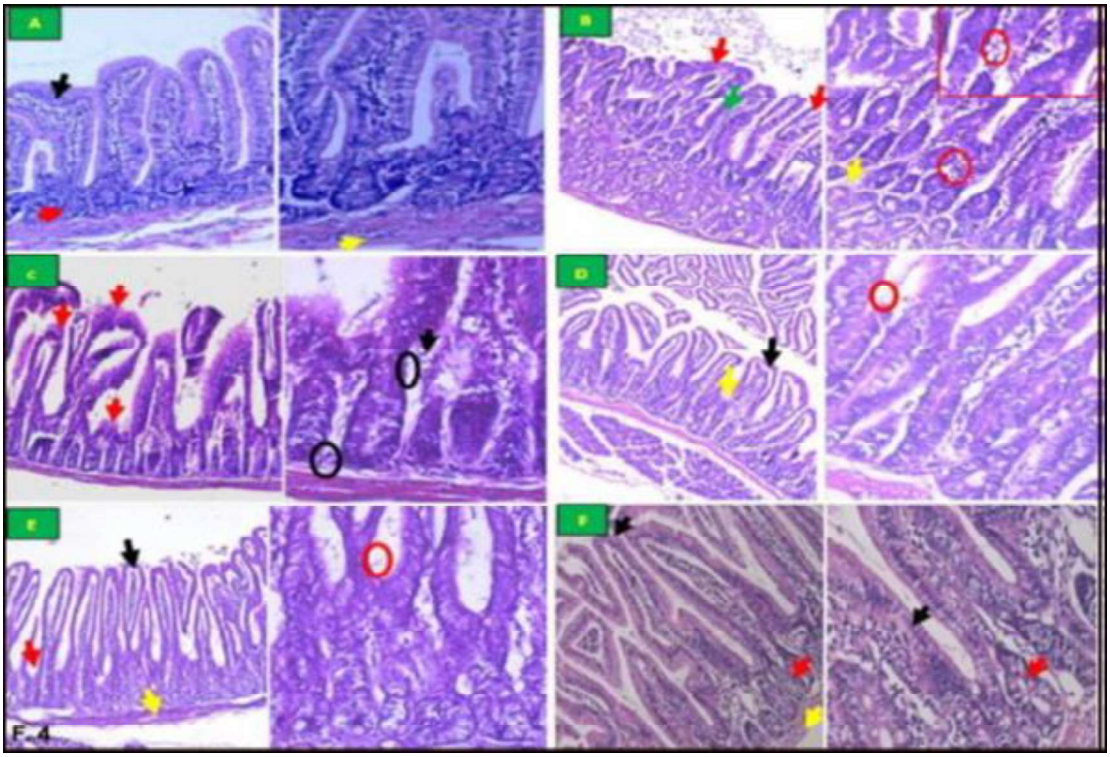
F. 1



F. 2



F. 3



F. 4