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Full Length Research Paper

Therapeutic effect of *Phoenix dactylifera* against cryptosporidiosis in immunocompromised mice

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Cryptosporidium infection can cause severe consequences in immunodeficient hosts, yet no effective drug therapy has been approved till now. Thus, it is increasingly necessary for evaluating new potential drugs against *Cryptosporidium* in immunocompromised patients. The strain of *Cryptosporidium* was identified using nested PCR and revealed *C. parvum*. The present work was carried out to evaluate the effect of aqueous extract of *Phoenix dactylifera* fruits against *C. parvum* in immunosuppressed mice. Then, the anticryptosporidial and anti-inflammatory effects were studied through evaluation of oocyst output, immunological parameter (INF- γ and IL-10). Very high statistically significant ($p < 0.001$) were seen post treatment with *P.dactylifera*, the percentage reduction of oocysts in faeces was 88.21%. Both cytokines INF- γ and IL-10 were increased before and after treatment with *P.dactylifera* when compared to control normal mice. Histopathological and electron microscopic examination revealed complete healing of intestinal mucosa after 21 days of treatment. Conclusion: *Phoenix dactylifera* is a promising anticryptosporidial global treatment without side effects on immunosuppressed host.

Keywords: *Phoenix dactylifera*, *Cryptosporidiumparvum*, IL-10, IFN- γ , immunosuppressed.

INTRODUCTION

Cryptosporidium spp. is an enteric parasite, oocysts-forming, apicomplexanprotozoa, which complete their life cycle both in humans and animals, through zoonotic and anthroponotic transmission, causing cryptosporidiosis. It has a worldwide distribution and in most surveys it is considered to be among the two major pathogen *Giardia lamblia*, and *Cryptosporidium parvum* causing diarrhoeal diseases (Putignani and Menichella, 2010).

Cryptosporidiosis is primarily characterized by

gastrointestinal symptoms such as profuse watery diarrhoea, fever, anorexia, weight loss, weakness, abdominal cramps, vomiting, inflamed joints (Hunter et al., 2004). The parasite affects the epithelial lining of the ileum, causing blunting of the microvilli and increased cellularity of lamina propria were observed focally in the distal jejunum, ileum and caecum. (Enemark et al., 2003).

The immune status of the host plays a critical role in determining susceptibility to infection with this parasite as well as the outcome and severity of cryptosporidiosis. In immunocompetent hosts infection is moderate and self-limited (Hunter and Nichols, 2002). However, in immunodeficient hosts including those with acquired

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immunodeficiency syndrome (AIDS), certain cancer patients undergoing chemotherapy and organ transplant recipients treated with drugs that suppress the immune system, infection can result in persistent, debilitating and possibly fatal diarrhoea (Hunter and Nichols, 2002).

Supportive therapy is a key component in the management of cryptosporidiosis. Potential anticryptosporidial agents have been screened, including natural products (Gaafer, 2012). *Phoenix dactylifera* (date) fruit was considered as one of the most important source of food for humans (Saddiq and Bawazir, 2010). Date fruits have anti parasitic properties against coccidiosis caused by *Eimeriapapillata* (Metwaly et al., 2012). Hot water fruit extract of *P. dactylifera* can also stimulate cellular immune system in mice (Karasawa et al., 2011).

In fact, Muslims believe that “*He who eats seven P. dactylifera every morning will not be affected by poison or magic on the day he eats them*” (Miller et al., 2003).

The present study has been designed to investigate the ameliorative effect of water extract of *P.dactylifera* fruits of the pathological change-mediated by the intestinal coccidian parasite, *Cryptosporidium parvum*.

METHODS

Mice

Thirty laboratory CD-1male mice aged 6–7 week with a weight range of 20-22 gm were obtained from Schistosoma biological supply program (SBSP) in Theodor Bilharz Research Institute (TBRI) Cairo, Egypt. The animal experiment was carried out according to the internationally valid guidelines and institution responsible for animal ethics [Schistosoma Biological Supply Program unit (SBSP) at Theodor Bilharz Research Institute].

The *Cryptosporidium* oocysts

Cryptosporidium oocysts were obtained from [The Animal Reproduction Research Institute (ARRI)] Giza, Egypt. Samples were concentrated by modified Formalin-ether sedimentation technique (Waldman et al., 1986) and then separated by Sheather's sugar floatation technique (Zeibig, 1997) then stained by modified Ziehl-Neelsen acid fast method (MZN) (John and Petri, 2006). Oocysts were suspended in phosphate buffered saline (PBS) with 0.01% Tween20, and stored at 4 °C in the presence of antibiotics (penicillin, streptomycin and amphotericin B) (Benamrouz et al., 2012).

Molecular studies

Part of frozen samples kept at -20°C were processed for detection of *Cryptosporidium* copro-DNA by analysis of COWP gene using Nested PCR (nPCR), and subjected for restriction enzyme cleavage to determine *Cryptosporidium* genotypes (Pedraza-Díaz et al., 2001).

Plant material

Fresh fruits of Siwa (*Phoenix dactylifera*. L) were obtained from a local market in Cairo, Egypt. Botanical identification of fruit was made in the Agricultural Research Center at Ministry Agriculture and Land Reclamation, Egypt, Cairo.

The preparation of the aqueous extract of *P. dactylifera* fruit

The date fruit was washed by Tap water then by distilled water. The fruit was manually separated from the pits and cut to small pieces then soaked in cold distilled water (1:3 ratio, weight to volume) the solution was filtered and kept for 48 hours at a temperature of 4°C, the methods were described previously by Al-Qarawi et al. (2004). The water extract was prepared freshly and given to the animals. Aqueous *P. dactylifera* extract was given orally in a dose of (4ml/kg) of aqueous extract of date fruit (20 mg/kg) for 21 consecutive days three times a day. The dose and the route of inoculation were selected on the basis of the previous studies (Saddiq and Bawazir, 2010).

Nutritional composition of date palm (*Phoenix dactylifera* L.) fruits

Total Phenols

The total phenolic compounds were estimated by Folin-Ciocalteu method according to Velioglu et al. (1998).

Total proteins

Protein content was measured by the Bio-Red micro assay as described by Bradford (1976).

Total amino acid

The total amino acid was measured according to Moor and Stein (1954).

Sugar content

Sugar content was determined as described by Shales and Schales (1945).

Experimental design

Two main groups; normal control group (I) uninfected non treated (ten mice) and Group (II) (20 mice) immunosuppressed group (mice were administered with 20 mg/kg/day dexamethasone sodium phosphate (Dex) intramuscularly 3 times per week for 20 consecutive days prior to oocysts inoculation (Rasmussen and Healey, 1992) were subdivided into two equal subgroups (ten mice each). Group (IIa) immunosuppressed infected non treated mice, the group (IIb) comprising infected immunosuppressed, treated with aqueous extract of *P. dactylifera* in a dose of (20 mg/kg) three times a day for 21 consecutive days. Groups each was inoculated with 10^2 *Cryptosporidium* oocysts in 200µl of phosphate buffered saline (PBS) per mouse by oral gavage (Benamrouz et al., 2012). The administration doses of the drug started after ten days post infection (PI).

Parasitological examination

Thirty one days after infection and administration of aqueous *P. dactylifera* extract fecal samples were collected from each infected mouse in clean, wide-mouthed containers with tight-fitting covers and homogenization in PBS. All stool samples were subjected to the modified Ziehl-Neelsen staining technique, examined by light microscope with (×1000) objective as described by John and Petri (2006).

Histopathological examination

The terminal 1 cm of the ileum was removed from each mouse, then fixed in 10% neutral formalin and embedded in paraffin. Sections of 4µm thickness were stained with Hematoxiline and Eosin stain (Drury and, Wallington, 1980).

Electron microscopic examination

Transmission electron microscope of the ileal intestinal region was done according to the technique of Grimaud et al (1980).

Determination of cytokine serum levels

Peripheral blood was aseptically collected from sacrificed mice. Sera were separated after centrifugation and stored at -20°C until analysis. In this study, (The Boster/Immuonoleader Mouse Cytokine ELISA Assay) kit (Boster Biological Technology Co., Ltd.) was used to quantify mouse (IL-10, IFN-γ). The assays were performed according to the manufacturer's protocol.

Statistical Analysis

Data were expressed as mean values \pm SD by the statistical software package SPSS (version 16.0). Continuous variables are presented, and frequencies with their respective percentages are given for categorical variables. Comparisons between groups were done using the Student's t-test. Percent inhibition compared to infected non treated oocysts was determined using the following equation: $[(\text{Infected immunosuppressed none treated} - \text{Treated}) / \text{Infected immunosuppressed none treated}] \times 100$. A p-value equal to or less than 0.05 was considered significant.

RESULTS

Cryptosporidium DNA uses a nested PCR procedure with the external primers BCOWPF and BCOWPR and the nested primers Cry-15 and Cry-9 to amplify a 553-bp region from the *Cryptosporidium* COWP gene. Using the restriction enzyme *Rsa* I digestion of (nPCR) product targeting COWP gene (PCR-RFLP) revealed the presence of genotype 2. The genotype 2 digestion products at 34, 106 and 410 bp (34 band is very small) Figure (1). The amplified DNA product of nested PCR (nPCR) revealed *Cryptosporidium* genotype is *Cryptosporidium parvum* (Figure.1)

Nutritional composition of date palm (*P. dactylifera*)

The chemical composition in date flesh was investigated, the experimental results presented in (Table 1 and 2). Protein content ranged about (1.9 mg/g). Total phenols in Siwa (Date fruits) water extract were (0.24 mg/g). In addition, the total amino acid content of fruits was found about (2.36mg/g) Table 1. The percentage total sugar of date was (71.7%). Reducing sugars were the major sugar component (50.17%), while non-reducing sugar was (21.53%) Table 2.

Oocyst shedding

The mean number and the percentage of reduction in number of *C. parvum* oocysts/g faeces three weeks post-treatment with extract of *P. dactylifera* fruits in immunosuppressed infected groups were seen in (Table 3).

Histopathological changes

Histological examination of sections of small intestines of mice in control group (I) revealed normal villous

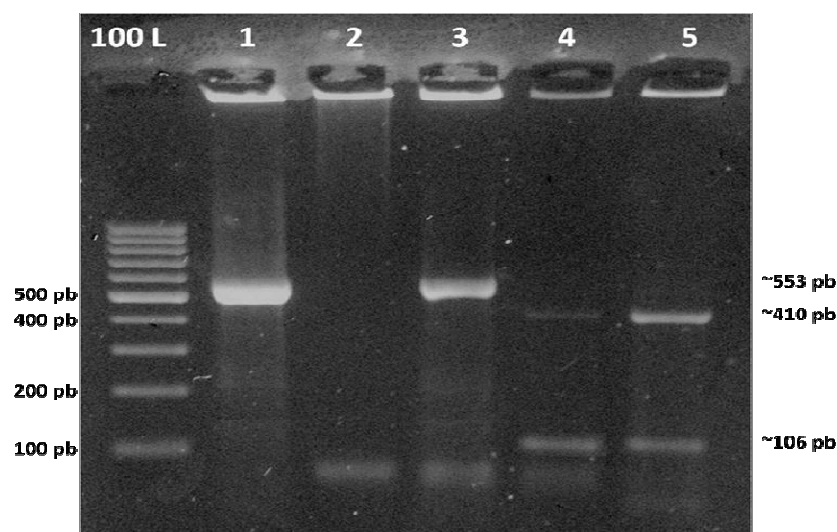


Figure 1. Agarose gel electrophoresis showing: L: 100 bp DNA molecular weight marker. Lane 1: nPCR products of the sample targeting COWP gene of *Cryptosporidium* at 553 bp. Lane 2: Negative control. Lane 3: positive control. Lane 4: RFLP products of sample after digestion with *RsaI* endonuclease with *C. parvum* genotype 2 digestion products at 34, 106 and 410 bp (34 band is very small, faint and difficult to see). Lane 5: RFLP products of positive control (*C. parvum*).

Table 1. Chemical composition (mg/ g fresh weight) of date flesh

Date Type	Protein content	Total phenols	Total amino acid
Siwa (Date fruits)	1.9	0.24	2.36

Values are expressed as Mean

Table 2. Percentage sugars content (mg/ g fresh weight) of date flesh

Date Type	Total sugar	Reducing sugar	Non-reducing sugar
Siwa	71.7%	50.17%	21.53%

The results are presented as Mean

architecture and normal brush border with no histopathological changes (Figure 2.A). Major histological changes were observed in group (IIa) associated with complete, severe villous atrophy changes. The presence of hyperplastic crypts and villus blunting associated with greater inflammation in the lamina propria (Figure 2.B). Few cells showed high nuclear cytoplasmic ratio, hyperchromatic nuclei (low grade dysplasias). No malignancy seen in this group (Figure 2.C). Also, *C. parvum* parasites in different stages were visualized in ileal villus epithelium and crypt glands (Figure 2.D). In treated group (IIb), section of small intestine revealed remarkable improvement in intestinal mucosa, with a preserved brush border, no ulceration and normal villous architecture. There was mild depletion of goblet cells and patchy inflammatory cellular infiltration of lamina propria with oedema. The ratio between villous and crypt was increased (Figure 2.E).

The ultrastructural examination of group (IIa) showed many stages of *C. parvum* life cycle. Longitudinal – shaped of cryptosporidial sporozoite with (apical region and dense granules) invaded intestinal epithelial cell and formation of a feeding connection to the host cell, known as the feeder organelle (Figure3.A). Developed meront type I, a rounded structure on the surface of host cell, parasitophorous vacuole (PV) closely surrounding the parasite membrane and microvillus material also became incorporated into the membrane (Figure3.B). Meront type 2 with merozoites, which will develop into male or female sexual stages, but still connected via stalk-like structure to the host cell. Microvilli were expressed in an irregular fashion and mainly seen accumulated around parasite, where the host cell microvilli (Figure 3.C). Group (IIb) revealed complete repair of the intestinal cell projection and the intestinal sub mucosa with striated border. Some tips of the microvilli are covered with glycocalyx and no *C. parvum* parasites were seen (Figure 3.D).

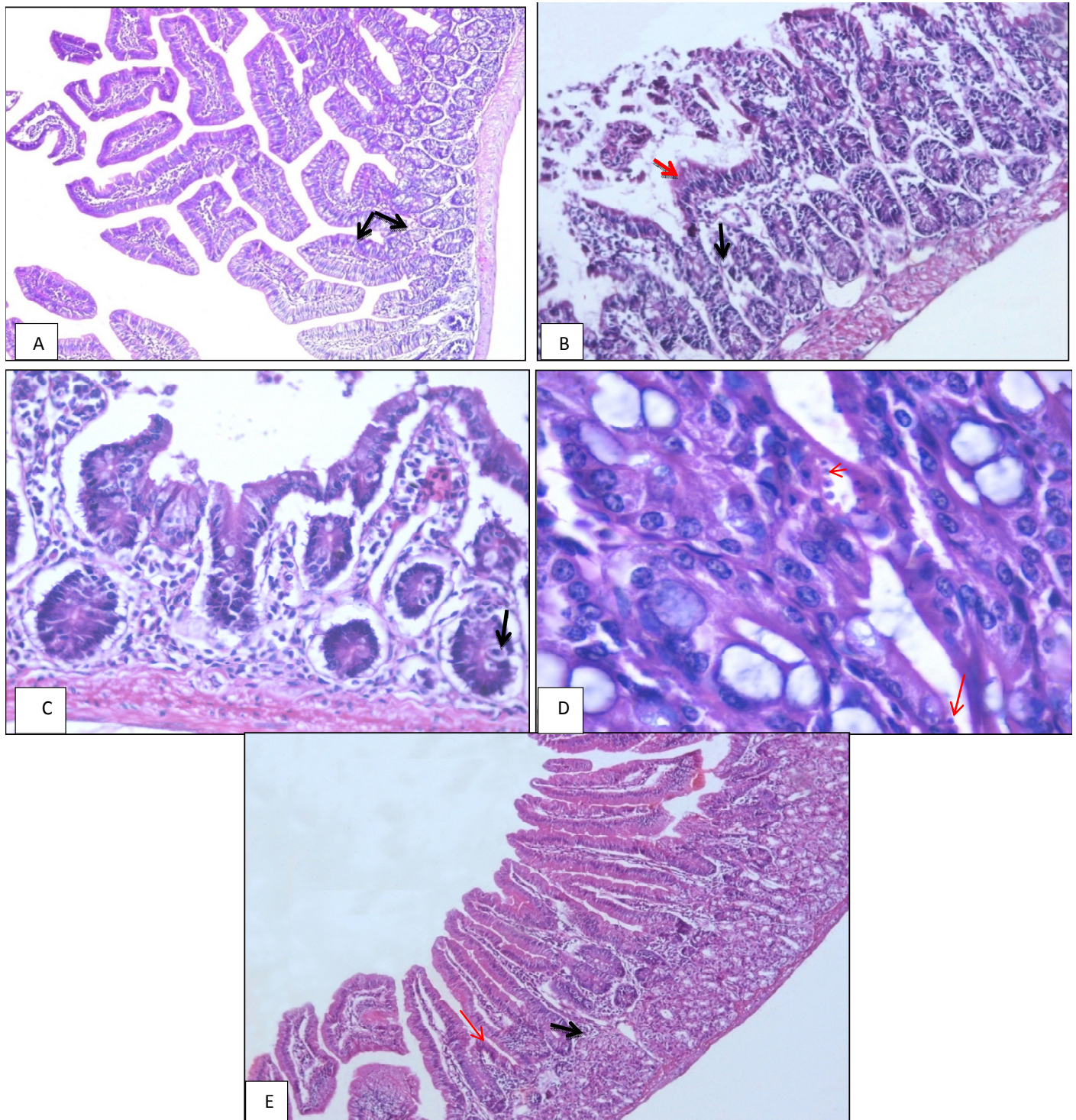


Figure 2. Section of small intestine Hematoxylin and Eosin staining: (A) control group (I) showing normal small intestine crypt villous (Black arrows) with normal mucosa (x 100), (B) group (IIa) showing severe villous atrophy with surface multi-form (Red arrow) and moderate exudation of mononuclear inflammatory cells in lamina propria (Black arrow). (x200), (C) low grade dysplasia (Black arrows) (x400), (D) presence of *C. parvum* parasites at different developmental stages (Red arrows). (x1000), (E) group (IIb) showing normal villous architecture (Red arrow), patchy inflammatory cellular infiltration of lamina propria with oedema (Black arrow) (x100).

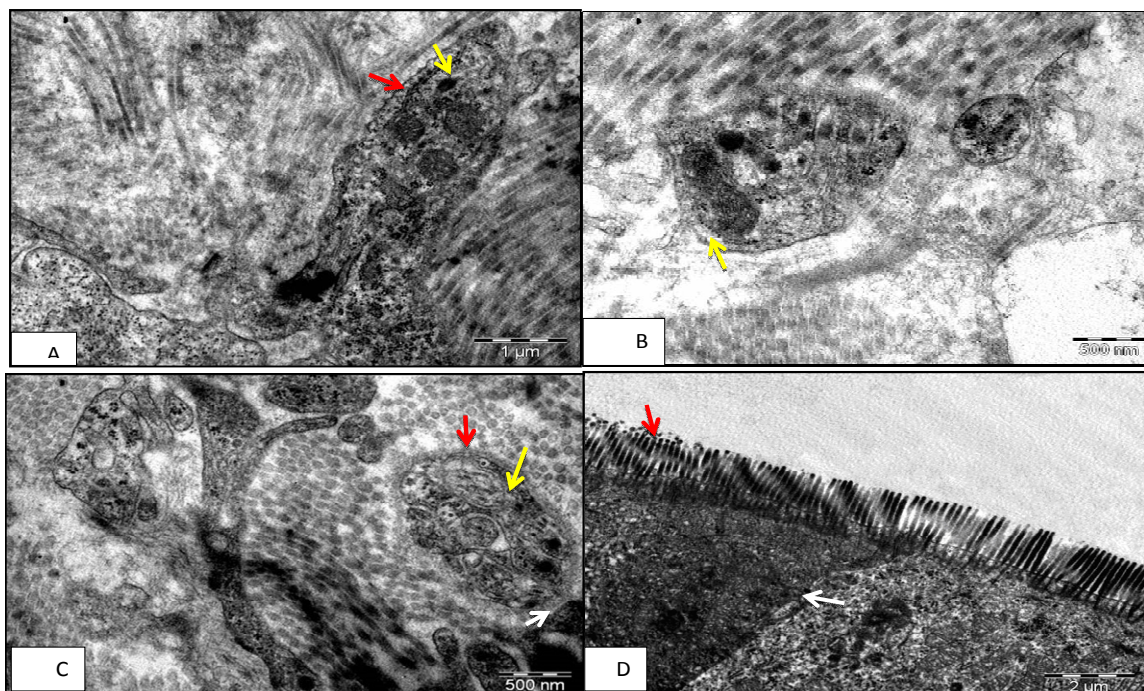


Figure 3. Electron micrograph of small intestine in immunosuppressed infected group (IIa) showing sporozoite (Red arrow) with dense granule (Yellow arrow), (B)meront Type I, parasitophorous vacuole (PV) closely surrounding the parasite (Yellow arrow), (C) meront type 2 (Red arrow) with merozoite (Yellow arrow). Meront type 2 however attached to host cells via a stalk-like structure (White arrow), (D) group (11b) two adjacent enterocyte (White arrow) with well-preserved brush border. Some tips of microvilli are covered with glycocalyx (Red arrow).

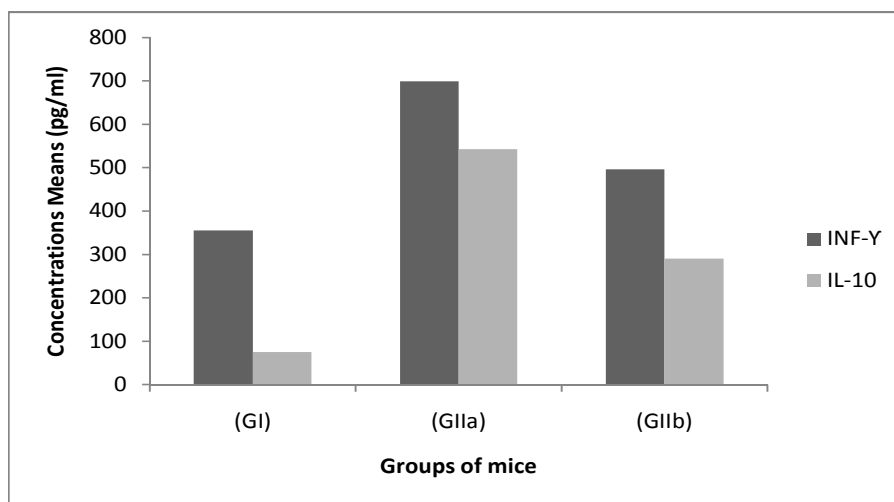


Figure 4. INF γ and IL-10 Cytokine levels, in immunosuppressed infected mice before and after treated with aqueous extract of *P. dactylifera* fruit compared with non-infected control mice.

Table 3. The mean values of number and the percentage of reduction of *Cryptosporidium* oocysts/ g faeces 21 days post-treatment in different studied groups.

Groups	(Mean \pm SD)	% Reduction in number of <i>Cryptosporidium</i> oocysts
GI	-----	----
GIIa	15973.33 \pm 2851.954	----
GIIb	1724.33 \pm 683.60	88.21**

GI: Control group, GIIa; Infected immunosuppressed, GIIb: Infected immunosuppressed aqueous date extract treated.

Mice serum cytokines

During the infection period, before treatment with *P. dactylifera* fruit, both IFN- γ and IL-10 circulating cytokines were increased in immunosuppressed infected mice when compared to control mice. IFN- γ mean value in group (IIa) was (699pg/ml), while in group (IIb) became (496 pg/ml) both groups showed higher increased when compared with control group (I) which (378 pg/ml) was. Mean level of IL-10 in group (IIa) was (543 pg/ml) then reached to (290pg/ml) in group (IIb). Also groups showed increase in the mean level of IL-10 before and after treatment when compare with control group (I) which was (75pg/ml) Figure 4.

DISCUSSION

Characterization of *Cryptosporidium* spp. by molecular methods is considered important for understanding the epidemiology of cryptosporidiosis (Hadfield et al., 2011). Large numbers of studies have suggested that *C. parvum*, *C. bovis*, *C. andersoni*, and *C. ryanae* are the most common species infecting calves (Zhang et al., 2013).

Several degrees of inflammatory changes were seen in the group infected immunosuppressed. Regarding the histopathological findings, the terminal part of the ileum was found to be the site with the heaviest burden of infection this result is in agreement with the findings of Certad et al. (2007) and Abdou et al. (2013). Low grade dysplasia was seen at the end of experiment but no malignancy was detected, these results were similarly reported by Abdou et al. (2013) who added that immunosuppressed mice infected with *C. parvum* developed intestinal dysplastic changes after 30 day post infection with no frank carcinoma seen.

This study demonstrates the first successful attempt to treat cryptosporidiosis using aqueous extract of *P. dactylifera* fruit. In traditional Egyptian medicine, *P. dactylifera* is listed in folk remedies for the management of diabetes, liver diseases and gastrointestinal disorders (Abdelaziz and Ali, 2014).

The obtained results of the chemical composition of date fruit showed that date fruits are rich in sugar. They contain proteins, amino acids and phenolic compounds. This result is in agreement with previous studies by (Al-Farsi and Lee, 2008; Assirey, 2015) who found that dates are rich source for nutrients as carbohydrates (44-88%), dietary fibers (6.4-11.5%), fats (0.2-0.5%) and proteins (2.3-5.6%). In addition there are 23 types of amino acids in date's proteins and some of them are not present in nutritious fruits like bananas, oranges and apples. Besides vitamin A, B1, B2 and nicotinic acid are also

constituents of dates (Al-Farsi and Lee, 2008; Assirey, 2015). Evaluation of the role of aqueous extract of *P. dactylifera* fruit (at dose 100 μ l three times a day for ten days), given to each mouse showed significant ($p < 0.001$) lowering of the shedding of *C. parvum* oocysts. Histopathological finding revealed an improvement in the form of healing of the intestinal mucosa, normal goblet cells, increase in the ratio between villous heights to crypt length and no ulceration seen. This is in agreement with studies which suggested that the aqueous extracts of the *P. dactylifera* fruit was effective in ameliorating the severity of gastric ulceration and gastrointestinal disorder (Al-Qarawi et al., 2005; Agbon et al., 2013). The *P. dactylifera* contain high amounts of anti-oxidant substances; it is possible that the mechanism of the gastro protective activity is via an antioxidant action (Al-Qarawi et al., 2005). The ultrastructural examination after treated with aqueous extract of *P. dactylifera* fruits showed healing of mucosa and striate brush border. No *C. parvum* stages at the surface have been seen. The *P. dactylifera* extract could be effective against extracellular stages promising (sporozoite, macrogamonts and merozoites) which could represent a susceptible drug targets INF- γ mean level and IL-10 mean level in treated group showed a increase. In addition the immunomodulatory effects of water extract of the *P. dactylifera* fruits were investigated in mice showing that number of IFN- γ , cluster of differentiation CD4, CD49b, IL-12 and CD11b cells was increased than normal after treatment (Karasawa et al., 2011). Many components were found in *P. dactylifera* fruit extract such as mineral "include boron, calcium, cobalt, fluorine, iron, magnesium, manganese, potassium, phosphorous, and zinc. These element with protein and amino acid and vitamin such as vitamin C, and vitamins B(1) , B(2) and vitamin A. Karasawa et al. (2011), reported that mice receiving oral treatment with polyphenols rich extracts from date palm tree, showed an increment of the immune cells, including natural killer (NK), macrophages and dendritic cells (DCs) in both Peyer's patches and spleen this might play important role in improvement immune function. Most Middle Easterners believe that seven *P. dactylifera* (date) a day, particularly in the morning on an empty stomach, can reverse the actions of any toxic material (Al-Qarawi et al., 2004).

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