Anti-*Cryptosporidium* efficacy of *Olea europaea* and *Actinidia deliciosa* in a neonatal mouse model

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Background
Cryptosporidiosis is caused by an opportunistic protozoan parasite *Cryptosporidium*. It may be life-threatening in immunocompromised individuals, children and the elderly. To date, no specific therapy has been proven to be effective against *Cryptosporidium*, which necessitates exploring for new therapeutics. This study evaluated the anti-*Cryptosporidium* therapeutic potential of two natural medicinal plants – *Olea europaea* (olive leaf extract) and *Actinidia deliciosa* (kiwi fruit pulp extract) – in four different groups of experimentally infected neonatal mice.
Materials and methods
Anti-*Cryptosporidium* efficacies of tested extracts were evaluated in four groups of age-matched neonatal Swiss albino mice parasitologically by detection of *Cryptosporidium parvum* oocysts and copro-DNA, using microscopy and nested PCR assay, as well as histopathological examination of their small intestines.
Results and conclusion
There was a 100% reduction in *Cryptosporidium* oocyst excretion in stool and copro-DNA of *O. europaea*-treated infected mice after 2 weeks of drug administration, whereas there was persistence of oocysts in the stool of *A. deliciosa*-treated mice until scarification. Obtained results make *O. europaea* a promising natural therapeutic for cryptosporidiosis, a scientific case that calls for further clinical trials to replicate this model in human individuals.

Keywords:
Actinidia deliciosa, *Cryptosporidium parvum*, neonatal mice, *Olea europaea*

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Introduction

Cryptosporidiosis is one of the most common waterborne protozoal diseases worldwide. This disease is caused by *Cryptosporidium*, a protozoan parasite that is highly infectious for humans and animals [1].

The genus *Cryptosporidium* consists of various species and genotypes that infect a wide range of hosts. *Cryptosporidium parvum* infects the epithelial cells of small intestine of mammals and causes acute short-term infections [2].

The infection may become chronic and life-threatening and can lead to death specifically in children, elderly and immunocompromised individuals [3].

Although many antimicrobial compounds have been tested for their efficacy against cryptosporidiosis in animals and humans, there is no effective, adequate or reliable chemotherapeutic treatment for *Cryptosporidium enteritis* [4].

Some antiparasitic agents such as paromomycin and nitazoxanide usually have temporary effects with relapses. *Olea europaea* [olive leaf extract (OLE)] and *Actinidia deliciosa* (kiwi fruit pulp extract) have well-known anti-inflammatory and antioxidant effects and still have been under investigation for their antiparasitic activity [5,6].

The aim of the present study was to evaluate the therapeutic efficacy of two natural medicinal plants *O. europaea* 'OLE' and *A. deliciosa* (kiwi fruit pulp extract) on experimental cryptosporidial infection in neonatal mice.

Materials and methods

Source of plant extract

Olive leave extract was used as standardized commercial vegetarian capsules containing 500 mg of 20% Oleuropin, with bitter glycoside, and ingredients including plant-derived capsule, magnesium stearate

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and silica. It is purchased from Nature's Way, USA. The manufacturer recommends it for its antiviral and antifungal effects, and for its supportive effects on the immune function.

Whole extract of kiwi's flesh, skin and seeds was used as commercial vegetarian source of vitamins and antioxidants in the form of 400 mg capsules. It was purchased from Swanson Premium, USA.

Parasite preparation

Cryptosporidium oocysts from naturally infected calves attending veterinary clinics in Faculty of Veterinary Medicine were identified by acid-fast (AF)-stained microscopic examination and characterized at the molecular level using nested PCR and PCR-RFLP, as described below, and all obtained parasites were *C. parvum* (Fig. 1). Positive stool samples were pooled, sieved and preserved in potassium dichromate 2.5% solution (wt/volume) at 4°C and used for infection of mice within 4 weeks. Oocysts were washed three times in PBS and counted with a haemocytometer; they were then diluted in distilled water to obtain 10^4 oocysts/ml and were used for infection of mice.

Experimental animal

A batch of 60 laboratory-bred neonatal male Swiss albino mice of CDI strain were used for the experiment. Mice were kept in well-ventilated cages in the animal house in Parasitology Department, Theodor Bilharz Research Institute and were provided with pelleted food and water. They were kept at room temperature (28+2°C) and away from direct sunlight. The cages were cleaned twice a week to ensure good sanitary conditions until required for use. The mice were allowed to adapt to the laboratory environment for 1 week before the experiment, and their stools were examined daily by direct wet saline smear and iodine before and after concentrations and then stained with AF stain to exclude the presence of parasites.

Experimental protocol

The Swiss albino mice used were divided into four groups (G1, G2, G3 and G4), each consisting of 15 mice. G1, G2 and G3 were infected with *Cryptosporidium* by intragastric inoculation of 10^4 *C. parvum* oocysts per mouse using an oesophageal tube. After 3 days from establishment of infection known by shedding of oocysts in stool, G1 and G2 were given an oral dose of 15 and 12 mg/kg orally from *O. europaea* and *A. deliciosa*, respectively, every other day for 2 weeks. The G3 mice were infected but nontreated (infected-untreated positive control), whereas the G4 mice were noninfected and nontreated (noninfected negative control).

Scarification

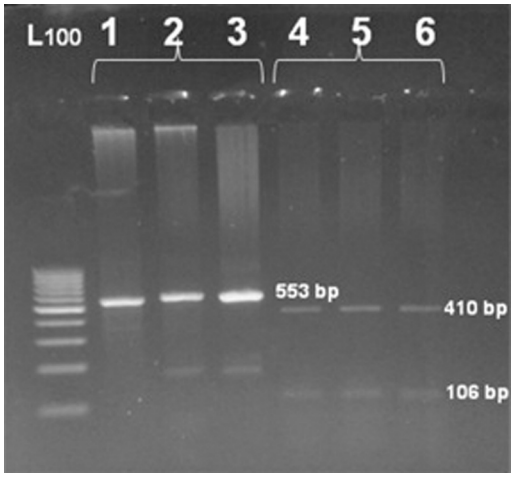
Scarification was done after cessation of treatment by rapid decapitation of all mice. Duodenal content was collected, stained with AF stain and examined microscopically for *Cryptosporidium* oocysts. Intestinal sections were dissected from individual mice, fixed in 10% formalin and subjected to histopathological studies.

Evaluation of the anti-*Cryptosporidium* efficacy of plant extracts

Coproscopic and copro-nested PCR assays

Stool samples were collected from all groups from the first day of treatment until the end of the experiment and were examined for detection of *Cryptosporidium*. Faecal smear from part of collected stool samples was stained with AF staining method before and after modified Ritchie's biphasic concentration [7], and examined microscopically for the presence of faecal *Cryptosporidium* oocysts. Part of each faecal specimen was frozen at −20°C for molecular studies. Genomic DNA was extracted from fresh-frozen faecal samples using spin column of FavorPrep stool DNA isolation Mini Kit (Favorgen Biotech corporation, Pingtung, Taiwan) according to the manufacturer's instructions. Extracted copro-DNA was amplified by nested PCR (nPCR) targeting COWP gene, using outer primers BCOWPF

Figure 1



Agarose gel electrophoresis for the DNA products. Lane L: 100-bp DNA molecular-weight marker. Lanes 1–3: Products of the nested PCR targeting COWP gene of *Cryptosporidium* at 553 bp; 1 is the positive control, and 2 and 3 are the used samples. Lanes 4–6: RFLP products 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); 4 is the positive control, and 5 and 6 are the used samples.

(5′-ACCGCTTCTCAACAACCATCTTGTCCTC-3′) and BCOWPR (5′-CGCACCTGTTCCCACT-CAATGTAAACCC-3′), which amplify a 796-bp fragment, and inner primers cry-15 (5′-GTAGATAA-TGGAAGAGATTGTG-3′) and cry-9 (5′-GGACT-GAAATACAGGCATTATCTTG-3′), which amplify a 553-bp fragment according to [8,9]. PCR products were digested by *Rsa*I restrictive enzyme (Fermentas UAB, Vilnius, Lithuania) and resolved by electrophoresis in 3.2% Metphore agarose gels containing ethidium bromide, and then fragments were visualized by ultraviolet light to determine *Cryptosporidium* genotype.

Part of the duodenal contents of scarified mice was smeared on a slide, AF stained and examined microscopically for *Cryptosporidium* oocysts. The other part was frozen at −20°C for nPCR-RFLP as faecal samples.

Coproscopy was performed in the Diagnostic and Research Unit of Parasitic Diseases, and nPCR assay was held in Lab of Molecular Medical Parasitology, Department of Medical Parasitology, Faculty of Medicine, Cairo University, Cairo, Egypt.

Histopathological studies

Ten small intestinal (ileal) sections of scarified mice of all groups were dissected and fixed in 10% formalin and embedded in paraffin. Next, all specimens were sectioned at 5 μm thick, stained with haematoxylin and eosin and examined to detect the histopathological changes that occurred because of *Cryptosporidium* infection and to assess the cure rates and the degree of healing of intestinal mucosa after drug administration. They were considered infected if at least one cryptosporidial developmental form was microscopically detected within an epithelial cell [10].

Ethical considerations

Experimental work with mice was conducted in accordance with the Egyptian National Animal Welfare Standards and was approved by the Ethics Committee of Theodor Bilharz Research Institute.

Data analysis

Data obtained from the study were statistically analyzed using statistical package SPSS version 17 (Chicago, IL, USA).

Results

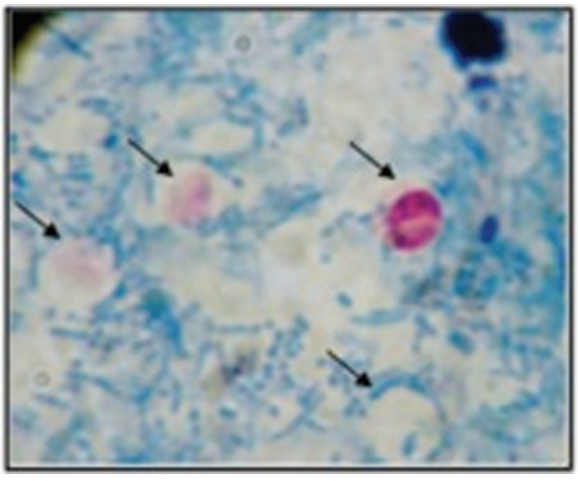
Parasitological and molecular assay results

Administration of OLE led to gradual reduction in *Cryptosporidium* oocysts, followed by the absence of *Cryptosporidium* oocysts and negative copro-DNA in stool and duodenal contents of G1 after completion of treatment (2 weeks) with significant difference ($P<0.001$). G2, treated with *A. deliciosa*, showed oocysts of Cryptosporidium in their stools and duodenal contents until scarification (Fig. 2). Examination of faecal smears of negative control group (G4) revealed the absence of oocysts all over the experiment (Table 1).

Histopathological results

The H&E-stained intestinal sections of the *Cryptosporidium*-infected control group (G3) revealed the presence of *Cryptosporidium* oocysts on the luminal surface of the epithelium lining of the villi, as small rounded organisms near the brush border of the villi with altered mucosal architecture, shortening, blunting and widening of the intestinal villi (Figs 3 and 4).

Figure 2



Cryptosporidium oocysts in stool.

Table 1 *Cryptosporidium* oocysts shedding in stool of all study mice groups in relation to time

Groups	Oocyst excretion in infected mice post-treatment and noninfected mice						
	2nd day	4th day	6th day	8th day	10th day	12th day	14th day
Infected *Olea europaea* treated (G1)	+	+	+	−	−	−	−
Infected *Actinidia deliciosa* treated (G2)	+	+	+	+	+	+	+
Infected nontreated positive control (G3)	+	+	+	+	+	+	+
Negative noninfected control (G4)	−	−	−	−	−	−	−

Intestinal sections of infected treated group with *O. europaea* showed slight or no pathological changes of the intestinal sections, which also regained their normal appearance with the absence of oocysts. Intestinal sections of infected treated group with *A. deliciosa* showed less histopathological changes with the presence of oocysts in comparison with the infected control group.

Discussion

Cryptosporidium infection is now considered one of the major causes of childhood diarrhoea and a key component of the cycle of infection and malnutrition, which is a major contributor to childhood morbidity and mortality worldwide. Until now, there is no satisfactory treatment for cryptosporidial enteritis, and instead a wide variety of medications and components have been tested; therefore, efforts are still needed to develop an effective drug [11,12].

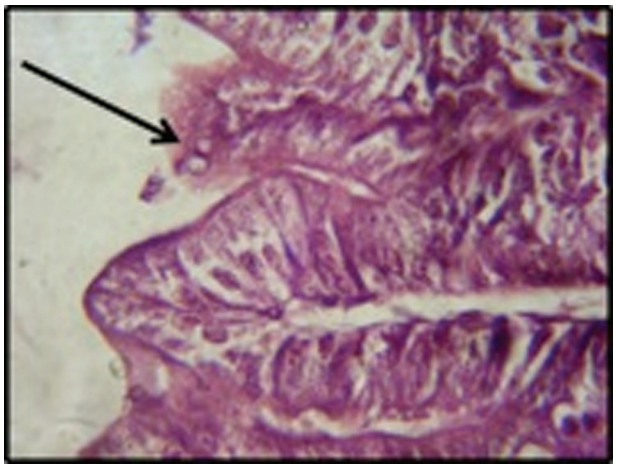
Most of the experimental cryptosporidial infections used rodent models to evaluate anti-*Cryptosporidium* efficacy of the potential agents; a majority of the experiments continue to use either the neonatal mice or the immunosuppressed adult mice because of availability, cost and applicable housing facilities for mice in most laboratories [13].

In the present study, neonatal mice have been chosen to assess the effect of the plant extracts on the infection in this critical age model as the high-risk group. In an experiment carried out by Leitch and He [14], the neonatal murine cryptosporidiosis model was used to test the role of reactive nitrogen compounds in limiting the severity of infection and limiting the age window during which it is possible to establish a low-grade infection. Shedding more light on the infection/malnutrition interaction risk in this age, Costa *et al.* [15] showed 20% additional weight loss when malnourished neonatal mice were infected with *C. parvum*. They reported higher faecal shedding of oocysts and weight loss despite treatment with nitazoxanide. In addition, children under the age of 1 year with Cryptosporidium infections fail to have catch-up growth that is typically observed with children infected at a later age [16,17].

Natural infection is usually initiated with oocyst ingestion, and thus oral inoculation was used in this study [18]. The number of oocysts needed to establish infection varies widely from 2^5 to 10^5 oocysts [19,20], depending on the species of the parasite and the experimental host. In the present study, 10^4 oocysts/ml were in the acceptable range of oocysts to create an experimental infection in neonatal mice.

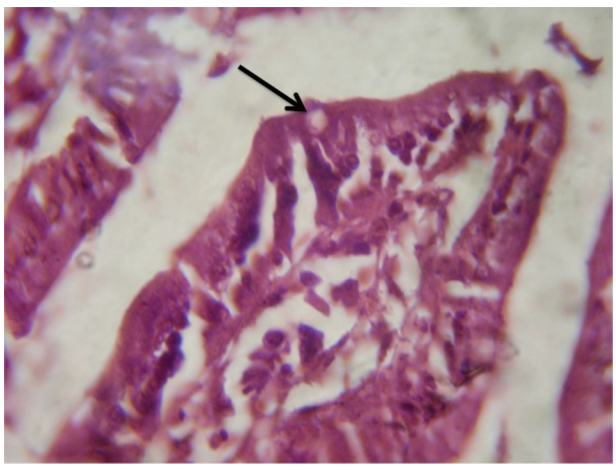
Products of some medicinal plants were used in traditional medicine because of their antimicrobial properties, and were recommended by the WHO to replace synthetic ones [21]. In the present experiment, plant extract administration continued every other day for 15 days. Obiad *et al.* [22] hypothesized that the dose and the days of treatment of the plant extracts may widely affect the number of oocysts shed in the faeces of infected mice and the day of disappearance of oocysts, and thus the doses of treatment must be continued for several days to allow antiparasitic effects of the active components of the drug. Perrucci *et al.* [23] found that Mangiferin was effective only at the end of 10 days of treatment. Moreover, histopathological examination of infected intestinal sections revealed altered mucosal

Figure 3



Section of Jejunum of infected control group showed two oocysts in the brush border.

Figure 4



Section of duodenum of infected control group showed one oocyst in the brush border.

architecture in certain areas, with minimal shortening and blunting of the intestinal villi with the presence of oocysts near the brush border. It was suggested that these changes might correlate with the number of infecting organisms [24]. The characteristics of histopatological changes in this study were similar to those in other experiments [25–28].

In the present study, *A. deliciosa* is one of the plants that are well known to be naturally containing cysteine proteinases, which is an active product against many helminthic infections [29]. The kiwi-extract-treated group continued shedding oocysts after finishing the treatment regimen, which proved that kiwi extract is not efficient in controlling *Cryptosporidium* infection. Moreover, the crudeness of the extracts, the dose and the oral route of administration might have also reduced the availability of sufficient active compounds [30].

However, *O. europea*, which has a well-known anti-inflammatory effect, was used in a dose equivalent to that daily recommended to maintain good health [31]. OLE has successfully cleared *Cryptosporidium* oocysts from stool, duodenal contents and intestinal sections of the infected group of mice with regaining of the normal intestinal architecture. In the same context, Reza *et al.* [32] have found that olive leaves are rich in polyphenols, such as oleuropein, apigenin-7-glucoside and luteolin-7-glucoside, and other ingredients, which are responsible for the functional properties especially antimicrobial activity [5,33]. The maximum antimicrobial activities were found in Oleuropein, which is an important constituent present in higher concentration in the leaves than in the fruits and other parts of the plant. Harp *et al.* [34] indicated that plant oils might compete for or block receptor sites on the surface of the ileum, thus leading to reduction in *C. parvum* colonization. The anti-*Cryptosporidium* effect may be caused by the presence of many individual phytochemical molecules or a synergy in-between.A Tunisian experiment reported that OLE of five different Tunisian olive varieties had antioxidant and leishmanicidal activities and relate that effect to the flavonoids present in the extract, which are broad classes of plant phenolics, which are known to possess a well-established protective effect against membrane lipoperoxidative damages with potent antiparasitic activity [35].

To find a safe applicable solution for such resistant threat, many studies [22,27,36,37] tested the effect of different crude plant extracts on experimental cryptosporidiosis, including garlic (*Allium sativum*), [27] onion (*Allium cepa*), Cinnamon (*Cinnamomum zeylanicum*) oils [36], Artemisia herba-alba and thymus vulgaris extract [37].

Conclusion

To the best of our knowledge, this is the first study to evaluate the anti-*Cryptosporidium* efficacy of these extracts. From the obtained data, *O. europea* has a promising effect on intestinal cryptosporidial infection that could be used as a naturally safe product for the preparation of a new therapeutic agent. However, it will be necessary to isolate and purify the bioactive components of OLE, and such result could be adapted in similar infections in animals or even humans.

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Authors contributed in every activity of the whole manuscript; idea of paper, study design, collection of materials, methodology, writing the paper and revising it. All authors undertake that it has not been published or submitted elsewhere.

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Conflicts of interest

There are no conflicts of interest.

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