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Effect of oregano essential oil and carvacrol on *Cryptosporidium parvum* infectivity in HCT-8 cells

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ABSTRACT

Cryptosporidium parvum is the second leading cause of persistent diarrhea among children in low-resource settings. This study examined the effect of oregano essential oil (OEO) and carvacrol (CV) on inhibition of *C. parvum* infectivity *in vitro*. HCT-8 cells were seeded (1×10^6) in 96-well microtiter plates until confluency. Cell viability and infectivity were assessed by seeding HCT-8 cell monolayers with *C. parvum* oocysts (1×10^4) in two modalities: 1) 4 h co-culture with bioactive (0–250 μg/mL) followed by washing and incubation (48 h, 37 °C, 5% CO_2) in bioactive-free media; and 2) 4 h co-culture of *C. parvum* oocysts followed by washing and treatment with bioactive (0–250 μg/mL) during 48-h incubation. Cell viability was tested using Live/Dead™ assay whereas infectivity was measured using *C. parvum*-specific antibody staining *via* immunofluorescence detection. Loss of cell viability was observed starting at 125 μg/mL and 60 μg/mL for OEO and CV, respectively. Neither OEO nor CV modulated the invasion of *C. parvum* sporozoites in HCT-8 cells. Treatment with bioactive after invasion reduced relative *C. parvum* infectivity in a dose-dependent manner to 55.6 ± 10.4% and 45.8 ± 4.1% at 60 and 30 μg/mL of OEO and CV, respectively. OEO and CV are potential bioactives to counteract *C. parvum* infection in children.

1. Introduction

Intestinal parasitic infection is considered a global public health problem by the World Health Organization (WHO), afflicting 3.5 billion people and causing clinical morbidity to approximately 450 million people globally [1]. Although it affects all ages and socio-economic status, parasitic infections are more prevalent in children (< 5 years) living in low-income settings, owing to inadequate sanitation, hygiene, and healthcare system and their less developed immune systems [2]. These infections alter the epithelial integrity and weaken the immune system in children resulting in reduced nutrient digestion and absorption, chronic gut inflammation, iron deficiency anemia, protein-energy malnutrition, and reduced growth and cognitive development [3].

Common parasites responsible for morbidity and mortality in children are helminths such as *Ascaris lumbricoides* (roundworm), *Trichiuris trichiuria* (whipworm), *Ancylostoma duodenale*, and *Necator americanus* (hookworms) and protozoans such as *Giardia intestinalis*, *Entamoeba histolytica*, *Cyclospora cayetanensis*, and *Cryptosporidium* spp. After rotavirus, *Cryptosporidium spp.*, mainly *C. parvum* (zoonotic) and *C. hominis* (anthroponotic), are the second largest cause of diarrheal disease and death in infants, posing 2 to 3 times higher risk of mortality [4]. These parasites follow a fecal-oral route of transmission. *C. parvum* oocysts are excreted in the feces of an infected host and are protected by a thick wall resistant to environmental factors, including most disinfection processes, making them highly resilient [5]. The infection starts with the release of sporozoites from oocysts in the intestinal tract where they attach and invade mucosal epithelial cells. In these cells, the entire endogenous development including asexual reproduction, gametogony and oocyst formation occurs [6]. *C. parvum* does not multiply outside of the host and completes all stages of its development (asexual and sexual) within a single living host. Thus far, *in vitro* models can only replicate the asexual cycle. Although cryptosporidiosis is self-limiting in healthy individuals, the infection can be life threatening among undernourished children with compromised immune systems.

There are a few compounds with some, albeit limited, anticryptosporidial activity including paromomycin, nitazoxanide, azithromycin in combination with paromomycin, roxithromycin, and protease inhibitors often used in highly active antiretroviral therapy

Abbreviations: OEO, Oregano Essential Oil; CV, Carvacrol; *C. parvum*, *Cryptosporidium parvum*; HCT-8, Human colon adenocarcinoma; PP1, Pyrazolopyrimidine 1; IC50, inhibitory concentration 50

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[7]. Currently, only one FDA-approved, moderately effective drug, nitazoxanide (Alinia®) [8], is available for treatment of *Cryptosporidium* infection. Due to increasing resistance and severity of side effects, new alternative bioactives are currently under examination [9].

Oregano Essential oil (OEO), also known as the Mediterranean miracle, *Sathra* in Ayurveda or Himalayan Marjoram in India, is known to possess antibacterial, antiviral, antifungal, antiparasitic and antioxidant activities [10]. The principal components responsible for the bioactivity of OEO are the small phenolic compounds carvacrol (CV) and thymol [10]. Although there is vast literature available on the activity of OEO and its constituents against bacteria, limited evidence exists on its efficacy and effectiveness against gut parasites, specifically *Cryptosporidium spp.* [10,11].

The objective of this study was to systemically examine the effect of OEO and its main monoterpene, CV, on the prevention of *Cryptosporidium parvum* invasion and infectivity using the HCT-8 cell model.

2. Materials and methods

2.1. Materials

OEO (*Origanum vulgare*) (origin: Turkey, steam extracted) was obtained from Oregano World, Hollywood, FL. Carvacrol (98% pure, 2-methyl-5-[1-methylethyl] phenol, liquid, M ≈ 150.2 g/mol, log extinction coefficient 3.262), Pyrazolopyrimidine 1 (PP1; positive control), RPMI 1640 medium, normal goat serum and sodium pyruvate were procured from Sigma-Aldrich (St. Louis, MO). Antibiotic-antimycotic and trypsin/EDTA were purchased from Gibco (Thermo Fisher Sci., Waltham, MA). Fetal Bovine Serum (FBS) and Sporo-Glo™ were obtained from Bio-West (Riverside, MO) and Waterborne Inc. (New Orleans, LA), respectively.

2.2. Preparation of C. parvum oocysts

Propagation, collection, and purification of *C. parvum* oocysts were conducted as previously described [12,13]. Briefly, oocysts were purified from homogenized and sieved feces by Sheather's sugar flotation and continuous sucrose density gradient centrifugation. Oocysts were washed and stored at 4 °C in 50 mM Tris buffer, pH 7.2 with 10 mM EDTA. Oocysts were used within 1 to 2 weeks of initial isolation from stored feces when viability remained above 80% as judged by excystation. All procedures involving animals were part of protocols approved by the University of Illinois Institutional Animal Care and Use Committee (IACUC).

2.3. HCT-8 cell culture

Human colon adenocarcinoma (HCT-8) cells obtained from ATCC (CCL244) were cultured in complete medium (RPMI 1640), supplemented with 2 g of sodium bicarbonate per liter, 2.5 g of glucose per liter, a final concentration of 10% FBS, 5 mL antibiotic-antimycotic solution, and 5 mL sodium pyruvate. Dissociating solution (2 mL; 0.25% Trypsin/EDTA) diluted with sterile PBS was used to subculture the cells in T-75 flasks, following incubation at 37 °C and 5% CO_2 for 15 min. Cells were used to seed 96-well plates for invasion and infectivity assays after grown to confluence within 2–3 days.

2.4. HCT-8 cell viability

Two-color dual parameter Live/Dead® cell viability assay (ThermoFisher Sci.) was used to assess the viability of cells after treatment with OEO and CV at different concentrations (0, 7, 15, 30, 60, 125, 250, 500 and 1000 μg/mL). Live cells were distinguished through the intracellular esterase activity. The green and red fluorescence were detected under phase contrast microscope using common green and red imaging filters used with FITC and Texas Red. HCT-8 cell viability assay was also conducted for both modalities to observe the viability after 4-h cell invasion assay and after 48-h growth inhibition assay. Secondary confirmation of cell viability was made through microscopic observation of cell deformations and damage at different concentrations.

2.5. C. parvum invasion and growth inhibition assay

The experimental design was based on previous studies for *in vitro* inhibition of parasitic invasion (modality 1) and infection (modality 2) of human cells using bioactives [12]. Two modalities were utilized to conduct the experiment as described below and shown in the graphical abstract. In either modality, plates with confluent monolayers of HCT-8 cells (1×10^6 cells/well) were used. Oocysts (0.5 mL, 5×10^4) were separately bleached with 40% NaOCl to facilitate oocysts excystation *in situ* on the cell monolayer. A positive control consisting of samples treated with PP1 was used at a level of 1.5 μM (0.179 μg/mL) to inhibit ~98% *C. parvum* infection with no effect on cells. Two empty wells were maintained between each concentration treatment horizontally and vertically to avoid cross contamination due to the volatile nature of the bioactives.

2.5.1. Invasion inhibition assay

In modality 1, oocysts (0.1 mL, 1×10^4) and cells were co-cultured in complete medium (0.2 mL, triplicate) containing several doses (0, 7, 15, 30, 60, 125, 250 μg/mL) of OEO or CV and incubated for 4 h at 37 °C and 5% CO_2. This was followed by rinsing the cell monolayers three times with PBS and reconstitution with bioactive-free media and 48 h incubation at 37 °C and 5% CO_2.

2.5.2. Growth inhibition/infection assay

In modality 2, oocysts (0.1 mL, 1×10^4) and cells were co-cultured in complete medium (0.2 mL, triplicate) and incubated for 4 h at 37 °C and 5% CO_2. Then, cells were washed with PBS three times followed by addition of 0.2 mL of several doses (same as before) of freshly prepared OEO or CV diluted in complete medium. Incubation continued for 48 h with or without complete medium replacement containing a fresh preparation of bioactives after 24 h.

2.5.3. Immunodetection

At the end of the growth inhibition assay, the medium was removed and the cells were fixed with methanol:acetic acid solution (9:1 v/v) for 2 min. Cells were rehydrated, permeabilized by two consecutive rinses with PBS containing 0.1% Triton X-100, blocked with 5% normal goat serum, and stained with Sporo-Glo™ overnight at 4 °C. Infectivity was assessed *via* immunofluorescence detection using phase contrast/fluorescent microscopy after rinsing twice with PBS, followed by imaging in water with a 20× objective as described previously [14]. Infected cells containing replicating parasites appeared as fluorescent clusters of infectivity or foci. The numbers of fluorescent particles within each focus per 20× field were determined by an automatic microscopic collection of nine fields per well of a 96-well plate. These nine fields were assembled into a single montage image for each well, representing 75% of the surface area of each well. The number of fluorescent particles in each montage was determined with Image J software, NIH, Bethesda, MD and expressed in fluorescent focus units (FFU). In both modalities, relative *C. parvum* growth was calculated at the doses described for each treatment dose using the control wells as 100% growth.

2.6. Carvacrol quantification

Quantification of CV in OEO was conducted *via* reverse phase HPLC with UV detection, which consisted of a Water 600 delivery system, 717plus autosampler, and 996 PDA detector (Milford, MA). Separation was conducted with a C18 Phenomenex Gemini column (5 μm,

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