Experimental Parasitology 181 (2017) 94–101

Contents lists available at ScienceDirect

Experimental Parasitology

journal homepage: www.elsevier.com/locate/yexpr

Effects of selected Indonesian plant extracts on *E. cuniculi* infection *in vivo*

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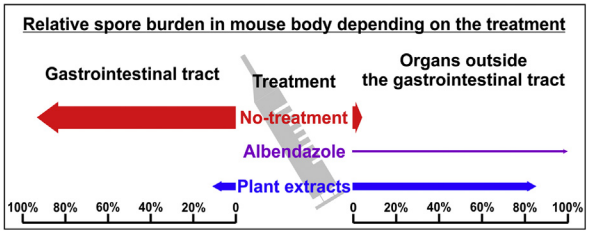
HIGHLIGHTS

- All plant extract tested exhibit to a certain extent antimicrosporidial effect.
- The antimicrosporidial effect of *Diospyros sumatrana* was comparable to Albendazole.
- The treatment led to infection shift towards organs outside gastrointestinal tract.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 5 January 2017
Received in revised form
1 June 2017
Accepted 31 July 2017
Available online 2 August 2017

Keywords:
Molecular detection
Antifungal
Plant extract
Encephalitozoon cuniculi
In vivo

ABSTRACT

The present study was conducted to evaluate the methanolic extracts from several plant leaves widely used in traditional medicine to cure digestive tract disorders and in the self-medication of wild animals such as non-human primates, namely *Archidendron fagifolium*, *Diospyros sumatrana*, *Shorea sumatrana*, and *Piper betle* leaves, with regard to their antimicrosporidial activity against *Encephalitozoon cuniculi* in immunocompetent BALB/c mice determined using molecular detection of microsporidial DNA (qPCR) in various tissues and body fluids of infected, treated mice.

Of the plant extracts tested, *Diospyros sumatrana* provided the most promising results, reducing spore shedding by 88% compared to untreated controls. Moreover, total burden per 1 g of tissue in the *D. sumatrana* extract-treated group reached 87% reduction compared to untreated controls, which was comparable to the effect of the standard drug, Albendazole.

This data represents the baseline necessary for further research focused on determining the structure, activity and modes of action of the active compounds, mainly of *D. sumatrana*, enabling subsequent development of antimicrosporidial remedies.

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http://dx.doi.org/10.1016/j.exppara.2017.07.014
0014-4894/

1. Introduction

Protozoan parasites of the genus *Encephalitozoon* are obligate intracellular organisms belonging to phylum Microsporidia which cause a wide spectrum of clinical manifestations, mainly in highly immunocompromised individuals, resulting in substantial morbidity and mortality (Didier and Weiss, 2011) despite the use of antiretroviral therapy (ART), which is not fully effective and less widely available in developing countries, where presentation with advanced human immunodeficiency virus (HIV) infection is common. Along with ART therapy, several drugs having antibacterial, antifungal or antiprotozoal properties have been used for control of microsporidiosis (Seddon and Bhagani, 2011; Weiss, 2014). Of these, Albendazole and fumagilin are widely used and are highly active against many microsporidia including all of the *Encephalitozoon* (Costa and Weiss, 2000; Seddon and Bhagani, 2011). However, recent studies have demonstrated that Albendazole (the treatment of choice) is only partially effective against *E. cuniculi*, which can survive in various organs of infected hosts and is able to reactivate from undetectable levels, spreading after the initiation of immunosuppression (Kotková et al., 2013; Lallo et al., 2013).

Thus, the fundamental problems with detecting *E. cuniculi* infection within hosts and subsequently evaluating the efficacy of treatment involve: (i) the ability of *E. cuniculi* to infect extra-intestinal parts of host bodies (Kotková et al., 2013; Koudela et al., 1994) and (ii) the intermittent shedding of very low numbers of spores in faecal/stool specimens during chronic infections (Kotková et al., 2013; Sak et al., 2010, 2011b). It can be assumed that previous studies evaluating efficacy of treatment only on the basis of a reduction of clinical signs and a decreased number of microsporidia spores in faecal samples could be misinterpreted (Sak et al., 2011b). Taken together, the availability of safe, effective and affordable treatments for microsporidiosis is highly needed. One possible approach is to investigate the use of plants that are widely employed in traditional medicine (mainly in communities with inadequate conditions of public health) or in the self-medication of wild animals such as non-human primates (Huffman and Hirata, 2004).

This study, for the first time, aims to determine whether extracts from pharmacologically active plants possess *in vivo* anti-microsporidial effects against the laboratory microsporidia model, *Encephalitozoon cuniculi*.

2. Material and methods

2.1. Ethics statement

The research complied with all legal requirements for research in Indonesia. The research permit was issued by RISTEK Kementarian Riset dan Teknologi. Permission to collect plant samples was obtained from LIPI – Lembaga Ilmu Pengetahuan Indonesia (Indonesian Institute of Sciences) and KKH - Kementerian Kehutanan Direktorat Jenderal Perlindungan Hutan dan Konservasi Alam.

All experimental procedures were conducted in accordance with the law of Czech Republic on the use of experimental animals, safety and use of pathogenic agents (Act No 246/1992 Coll., on the protection of animals against cruelty). The study design, including the number of animals used and the possibility of animal death without euthanasia due to experimental intervention, was approved by the Ethical Committee at the Institute of Parasitology, Biology Centre of the Czech Academy of Sciences, State Veterinary Administration and Central Commission for Animal Welfare under protocol no. 073/2010.

2.2. Experimental animals

Eight-week-old BALB/c mice (Charles River, Sulzfeld, Germany) were housed in plastic cages with sterilized wood-chip bedding situated in flexible film isolators (BEM Znojmo, the Czech Republic) with HEPA filters. All mice were supplied with a sterilized diet (TOP-VELAZ Praha, the Czech Republic) and sterilized water *ad libitum*.

2.3. Parasite

The spores of *E. cuniculi* strain EC2 were originally isolated from a dexamethasone-treated laboratory mouse (Koudela et al., 1993) and were grown *in vitro* in Green monkey kidney cells (VERO, line E6) maintained in RPMI-1640 medium (SIGMA-Aldrich, St. Louis, MO) supplemented with 2.5% heat-inactivated foetal bovine serum. Spores were isolated and purified from cells by centrifugation over 50% Percoll (SIGMA) at 1100 × *g* for 30 min and washed three times in sterilized deionized water before storing in sterilized deionized water supplemented with antibiotics (SIGMA, 100 U/ml penicillin, 100 μg/ml streptomycin, and 2.5 μg/ml amphotericin B) at 4 °C. Spores were washed in sterilized deionized water before use.

2.4. Plant extract preparation

Leaves of *Archidendron fagifolium* (Miq.) I. C. Nielsen (Fabaceae), *Diospyros sumatrana* Miq. (Ebenaceae), *Shorea sumatrana* Symington (Dipterocarpaceae) and *Piper betle* L. (Piperaceae) were collected from their natural habitat on Sumatra. Leaves were air dried in shade and dehydrated by lyophilisation (freeze-drying). Dried plant material was transported to the Czech Republic for further processing. To make the preparations, leaves were first ground in liquid nitrogen and then the methanolic fraction was extracted from coarse, dried powder of each particular plant separately using 10 mL of methanol. After 16 h of extraction (overnight) at −20 °C, the resulting homogenates were centrifuged (4 °C, 20 min); the sediments were re-extracted for 1 h in the same way and centrifuged again. These two supernatants were pooled and dried in a vacuum at 35 °C. The lyophilised extracts were stored in sealed glass tubes at −20 °C. Prior to use, the lyophilised extracts were first dissolved in 100 μl DMSO and adjusted with sterile dH_2O to a final concentration of 0.5% DMSO.

2.5. Experimental design

Prior to infection, all animals were screened for the presence of *Encephalitozoon* spp. using molecular tools as described below. Twenty-one mice were infected perorally with 10^7 *E. cuniculi* spores in 0.2 ml of sterilized dH_2O by intragastric gavage. Faecal samples from three randomly selected mice were obtained daily from the 1st to 20th day post infection (DPI), weighted with 0.0001 g accuracy, and stored at −20 °C prior to DNA isolation. On the twenty-first DPI, mice were divided into groups consisting of three animals each.

Five groups of mice were treated daily for 14 days perorally by intragastric gavage with one of the plant extracts (12.5 mg of original plant dry weight/200 μl dH_2O/mouse/day) or albendazole (Aldifal; Mevak Nitra, Slovak Republic, 0.2 mg/200 μl dH_2O/mouse/day) starting from the 21st DPI. One group of mice was treated with 200 μl of 0.5% DMSO in dH_2O/mouse/day (a negative control) and one group of mice remained untreated (200 μl dH_2O/mouse/day) and served as an untreated control. Faecal samples from all mice were individually obtained daily from the 21st DPI, weighted with 0.0001 g accuracy, and stored at −20 °C prior to DNA isolation.

Mortality and morbidity were recorded daily. All mice were