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Research paper

The antiparasitic activity of avenacosides against intestinal nematodes

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ABSTRACT

Avena sativa L., 1753 (Poaceae) is used as feed for livestock and as a crop rotation agent. The purpose of the study was to examine the molecular mechanisms behind the antihelminth activity of the oat saponins avenacoside B (AveB) and 26-desglucoavenacoside B (26DGAveB) by evaluating their effect on *Heligmosomoides bakeri*, a parasitic nematode of mice. The avenacosides AveB and 26DGAveB were separated and purified from *A. sativa* green leaves, and their mycotoxic activity was confirmed against the fungus *Trichoderma harzianum.* The anti-nematode activity of the avenacosides was measured by egg hatching assay. In the surviving L3 larvae exposed to avenacosides, the expression of CED-9, a protein of the apoptosis pathway, was identified by Western blotting. The protein profile of L3 larvae was monitored by High Performance Liquid Chromatography (HPLC). The action of saponins on glycoprotein pump (Pgp) activity in L3 larvae was compared to that of the pump blocker Verapamil (VPL). A mouse model was used to measure the infectivity of L3 larvae exposed to AveB and 26DGAveB, and the outcome of the immune response.

Both compounds induced morphological changes in larvae and blocked Pgp activity; however, only 26DGAveB provoked expression of CED-9. The infected mice displayed changes in the molecular pattern of larval proteins and enhanced IL-4 production, indicating that avenacosides reduced the infectivity of *H. bakeri* larvae. In avenacosides, the residue without glucose at the C26 position demonstrated greater anti-nematode activity. Our findings indicate that *A. sativa* compounds are natural products with anti-parasitic activity.

1. Introduction

Plant-derived compounds, particularly those of plants used as natural food products, may be used to treat parasitoses and generally improve the health of both humans and animals. Saponins are one of many groups of plant compounds that may have beneficial antibacterial, antiprotozoal and antitumor activities (Avato et al., 2006; Francis et al., 2002; Vincken et al., 2007).

The biological activity of saponins is derived from their amphiphilic nature (Lorent et al., 2014). They can cause harmful effects on pathogens by irreversibly binding to the cell membrane, thus increasing its permeability (D'Incao et al., 2012; Price et al., 1987). Our previous study found that triterpenoid saponins affected the development of free-living stages of *Heligmosomoides bakeri*, a parasitic nematode of the mouse intestine: a dysfunction in P glycoprotein (Pgp), the major membrane transporter for xenobiotics and a substrate of multidrug resistance protein-1, was detected by rhodamine 123 retention assay (Doligalska et al., 2011). Rhodamine, a substrate of Pgp, is a cationic dye that accumulates into the mitochondrial matrix based on the electric potential across the inner mitochondrial membrane. (Eytan et al., 1997). The activity of functional ATP-Binding Cassette transporter (ABC transporter) can be detected using specific inhibitors such as verapamil (VPL), which simultaneously displays activity as a substrate and an inhibitor toward Pgp (Bellamy, 1996). Among the saponins, steroidal glycosides are also of special interest, as their biological activity is manifested not only against plant parasites, but also against other invertebrates, including the free-living stages of animal and human parasites (Chitwood, 2002; Habtamu and Negussie, 2014).

The oat, *Avena sativa* L. 1753, has been cultivated for more than 4000 years and is today found worldwide. It is an important food product which serves as a rich source of proteins, as well as of a number of minerals, lipids, β-glucans and polysaccharides (Singh et al., 2013). *A. sativa* has also been used in traditional medicine since the 12th Century, with the oat herb, harvested before flowering, being used in herbal tea, in aqueous or ethanolic extracts, or as expressed juice. In agriculture, the white oat is employed in the sustainable management

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of plant parasites (Marini et al., 2016; Marshall et al., 2013) and Saia oat has been shown to control lesion nematodes (Inomoto et al., 2010; LaMondia et al., 2002; Townshend, 1989). Crude extracts of oat shoots with a saponin fraction containing 26-desglucoavenacoside B have also been found to inhibit the fungal growth of *Pyrenophora* species (Bahraminejad et al., 2008).

Young oat leaves act as a source of two steroidal glycosides, avenacosides A and B (AveA and AveB, respectively), which are derivatives of nuatigenin [22,25-epoxy-(20S)(22S)(25S)-furost-5-en-3β,26-diol]. Avenacoside A is 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-[β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside, the 26-*O*-β-D-glucopyranoside of nuatigenin; Avenacoside B is 3-*O*-[α-L-rhamnopyranosyl-(1 → 4)]-[β-D-glucopyranosyl-(1 → 3)-β-D-glucopyranosyl-(1 → 2)]-β-D-glucopyranoside, the 26-*O*-β-D-glucopyranoside of nuatigenin (Tschesche and Schmidt, 1966; Tschesche and Wiemann, 1977); chemical compounds: Avenacoside B (PubChem CID: 71581002), 26-desglucoavenacoside B (PubChem CID: 71768106).

The total concentration of AveA, AveB and their desglucosides has been found to be 0.2 mg/100 mg fresh oat leaves (Kesselmeier, 1982). These glycosides are localized in the vacuole, and are believed to be preformed compounds which demonstrate activity against fungal infections; oat leaves contain a very active and specific β- glucosidase, known as avenacosidase, which immediately converts AveA and AveB into the antifungal 26-desglucoderivatives (26DGAve) upon cell damage (Gus-Mayer et al., 1994; Kim et al., 2005; Pecio et al., 2013). The toxic mechanism of these saponins against fungi has been attributed to their lytic activity: when the saponins complex with membrane sterols, pores are formed, resulting in a loss of membrane integrity (Morant et al., 2008; Osbourn, 1996). It is possible that avenacoside may also possess lytic activity against nematode cell membranes, and apart from the clear anti-parasitic potential of such discovery, such knowledge of avenacoside activity would further elucidate the mode of action of oat-derived substances.

Unfortunately, relatively few studies have examined the anti-nematode activity of *A. sativa*. The aim of the present study was to determine whether AveB and its derivative 26DGAveB differ with regard to their effect on the development and infectivity of the nematode *H. bakeri* in mice. It also examines the potential mechanisms behind the anti-nematode activity of avenacoside and 26-desglucoavenacoside in a mouse, a laboratory model of gastrointestinal nematode infection in ruminants and hookworm in humans. The results indicate that *A. sativa* may serve as a potential source of antiparasitic drugs.

2. Materials and methods

2.1. Evaluation of raw oat juice activity against nematode

Plant material: Oat seeds (*Avena sativa* L., www.theplantlist.org), purchased at a local market, were sown on several layers of moistened wood-wool and germinated at 20 °C in the light: intensity *ca* 3000 lx; photoperiod 16 h/day (Kalinowska and Wojciechowski, 1987). The oat was grown for 10 days, then its leaves were pressed in a manual glass grinder. The leaked juice was stored in a black glass bottle and left overnight in 4 °C, then filtered through filter paper.

The parasite: *Heligmosomoides bakeri*, a nematode of laboratory mice is maintained for almost 40 years in the Department of Parasitology University of Warsaw. The nematode was a gift from Prof. Jerzy Behnke (Nottingham University, UK) and has since been maintained by monthly passage in adult mice. Five female C57Bl6 mice were infected with *H. bakeri*. After 28 days of infection, fecal material containing nematode eggs was collected and used for culture (Keymer and Hiorns, 1986). The infective larvae were harvested after seven days of culture, washed with distilled water and stored for two weeks at 4 °C.

Larval motility test: The motility of *H. bakeri* third-stage larvae (L3) were examined in the presence of decreasing dilutions of raw oat juice. A twofold dilution series was created of the obtained *A. sativa* raw leaf juice in distilled water: A series of test ubes was set up, 2 mL of raw juice was added to the first tube, and 1 mL of distilled water added to the other tubes. Next, 1 mL was taken from the first tube, added to the second tube and vortexed. Another 1 mL was taken from the second tube, passed to the third tube, vortexed, and so on, to obtain a dilution series from 0 to 1:1024. A 900 µL volume of each *A. sativa* leaf juice dilution, including the pure juice, was poured into each well of a 24-well flat-bottom polystyrene plate (Costar, Corning, USA). The larvae were suspended in distilled water, and then added to each well of the plate at a concentration of 200 larvae in 100 µL. Each dilution was tested in triplicate. The half maximal inhibitory concentration (IC_{50}) of oat juice was determined after 18 h of incubation at 21 °C in the dark. Sinusoidal movement was stimulated through exposure of larvae to microscope light for five minutes and shaking the plates manually. Larvae kept in distilled water were used as a control sample in the test. All larvae were then counted under an inverted microscope (Leica, Leitz DM IL) at 100 x magnification and the results expressed as the percentage of motile larvae.

2.2. Isolation and purification of avenacosides and 26-desglucoavenacosides

The avenacosides were isolated from 10-day-old seedlings. The material was dried at 40 °C and then subjected to extraction for six hours in Soxhlet apparatus with diethyl ether; the fraction which contained less polar lipids was discarded. Defatted material was then subjected to a five-hour extraction with methanol. The MeOH extract was partly evaporated on a rotary evaporator and slowly added to five volumes of cold (4 °C) ethyl acetate. The obtained suspension was kept overnight at 4°C and then centrifuged at 3000 x *g*. The supernatant was discarded while the sediment was washed twice with cold ethyl acetate (three volumes) and dried under vacuum. This crude avenacoside fraction (cAves) contained AveA and AveB, as well as a small amount of 26DGAves.

The crude avenacoside fraction was hydrolyzed with avenacosidase from the cytosolic fraction (105,000 x *g*) of fresh oat leaves (Kalinowska and Wojciechowski, 1987). Hydrolysis was performed at 25 °C for 24 h on a rotary shaker (0.5 x *g*) in incubation medium containing an aqueous solution of cAves (20 mg/mL) and avenacosidase (1 mg/4 mg cAves). The reaction was terminated by adding *n*- BuOH (3 x extraction), and the extract was washed with water. The buthanol fraction containing crude 26DGAves was then evaporated to dryness.

Partial separation and purification of Aves and 26DGAves were performed by Solid Phase Extraction (SPE, Oleszek, 2002) on Supercline Supelco LC-6 (6 mL; 0.5 g) and LC-18 columns (12 mL; 2 g) developed in step (every 5% change) with a solvent gradient of 100% water → 100% methanol. Pure Aves and 26DGAves were then obtained using a series of preparative TLC (Thin Layer Chromatography) developed on SiO_2 in the solvent mixture: $CHCl_3$: MeOH: H_2O (70:35:6) and $CHCl_3$:MeOH (85:15) (Kalinowska and Mielecki, unpublished results). PubChem CID for Avenacoside B: 71581002 and for 26-desglucoavenacoside B: 71768106.

2.3. The mycostatic activity

The mycostatic activity of avenacosides and 26-desglucoavenacosides was evaluated against *Trichoderma harzianum* (obtained from dr M. Wrzosek, Department of Molecular Phylogenetics, University of Warsaw) culture on potato-dextrose agar. The leaking of amino acids has been shown in liquid cultivars (3% Sabourand dextrose medium). Hyphae (1 g) were incubated in 5 mL of 6% mannitol with or without 153 µM Aves for 24 h at 30 °C. The suspensions were centrifuged (10 min, 3000 x *g*) and the amino acid concentration of the supernatants was measured. The reaction mixture consisted of 1 mL supernatant and 1 mL ninhydrin reagent (100 mL of the reagent contain 400 mg ninhydrin, 400 mg cadmium acetate dihydrate, 25 mL 4 M