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SHORT COMMUNICATION

Plant extracts as natural amoebicidal agents

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Abstract Strains of *Acanthamoeba* sp. constitute a factor contributing to the occurrence of chronic granulomatous amoebic encephalitis, keratitis, pneumonia, as well as inflammations of other organs. Treatment of these diseases is very difficult and not always effective. A majority of these infections have been fatal. The aim of our study was to examine the amoebicidal or amoebistatic activity of plant extracts from *Rubus chamaemorus*, *Pueraria lobata*, *Solidago virgaurea* and *Solidago graminifolia*. For the purpose of isolation of pharmacologically active substances, we used the aboveground parts of plants, together with flowers, roots and leaves. It was established that extracts from *S. virgauera*, *P. lobata* and *R. chamaemorus* displayed chemotherapeutic properties in vitro in concentrations of approximately 0.01–0.05 mg extract/mL, i.e. in concentrations of 0.350 μg/mL expressed in ellagic acid for *R. chamaemorus* and 0.053 μg/mL expressed in puerarin for *P. lobata*. Therapeutic index values is 3.5–20. As a result of in vivo experiments, it was found out that, following therapy using the extracts, animals infected with *Acanthamoeba* sp. survived for an extended period (2.5–3 times longer). It was determined that plant extracts may be used both externally and internally in the case of a combined therapy for acanthamoebiasis. The tested extracts are not toxic for animals.

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Introduction

Free-living amoebae of the genus *Acanthamoeba* constitute an aetiological factor influencing chronic granulomatous amoebic encephalitis (GAE), *Acanthamoeba* keratitis (AK), amoebic pneumonitis (AP), as well as changes occurring in other human and animal organs. Treatment of *Acanthamoeba* infections is always very difficult and not always effective. Apart from antibiotics, which prevent further consequences of tissue damage, use is made of compounds that are amoebicidal or amoebistatic. These substances usually used for disinfection are frequently strongly irritating and toxic for the host (Kitagawa et al. 2003; Seal 2003).

The aim of the present study was to investigate the amoebicidal or amoebistatic effect of plant extracts obtained from *Rubus chamaemorus*, *Pueraria lobata*, *Solidago virgaurea*, *S. graminifolia*, *Eryngium maritimum* L. and *E. planum* L. on the growth and development of free-living amoebae of the genus *Acanthamoeba*.

Materials and methods

The plant material used to isolate pharmacologically active substances comprised the aboveground parts of plants, including flowers of common goldenrod *S. virgaurea* L. and grass-leaved goldenrod *S. graminifolia* (L.) Elliott. (Asteraceae), the roots of kudzu *P. lobata* (Willd.) (Fabaceae) and the leaves of cloudberries *R. chamaemorus* L. (Rosaceae).

Herbarial specimens of the researched plants are kept at the Department of Pharmaceutical Botany of Poznan University of Medical Sciences.

Each batch of dried and fragmented plant material (approximately 10.0 g) was extracted three times for 1 h in 150 mL of methanol. The combined methanol extracts were filtered off and dried in a vacuum on a water bath at a temperature under 40°C. Research into therapeutic properties was performed on dry extracts of each plant species, which were dissolved in distilled water.

The tested plant extracts underwent a phytochemical analysis aimed at establishing the presence of flavonoids in *S. virgaurea* and *S. graminifolia*, ellagic acid in *R. chamaemorus* and isoflavones in *P. lobata*.

Phytochemical analysis of extracts was performed using column chromatography, thin-layer preparative chromatography and high-performance liquid chromatography (HPLC; Krawczyk et al. 2003).

The influence of therapeutic substances obtained from plants was tested in vitro on strain 309 *Acanthamoeba castellanii* (pathogenic for mice, isolated from the environment; Kasprzak and Mazur 1972), and on the "Ograbek" strain *Acanthamoeba* sp. (pathogenic for humans, isolated for *Acanthamoeba* keratitis—own, previously undescribed strain).

The amoebae strains were axenically cultured on a liquid medium Bacto-Casitone+horse serum described by Červa (1966, 1969). Plant extracts were added to the axenic culture of amoebae (5×10^4 cells/mL) in the following concentrations: 0.01, 0.05, 0.1, 0.5, 1.0 and 1.5 mg/mL. The increase or decrease in the number of amoebae was checked at 24-h intervals using a Thoma haemocytometric chamber. The control group was a culture of amoebae without extracts. The IC_{50} coefficient refers to the lowest concentration of the researched substance that halts the growth of amoebae in 50%.

The pathogenic properties of amoebae were tested by infecting 2-week-old white mice of the BALB-c strain using the procedure described by Kasprzak and Mazur (1972) and Mazur (1984).

Research into the therapeutic influence of plant extracts on the progress of the infection and survival time of animals consisted in the daily provision to the intraperitoneally infected mice, over a period of 3 days, of sterile solutions of plant extracts in concentrations ranging from 0.1 to 0.5 mg of dry extract per 1 g of body mass. The extracts were dissolved in normal saline with a small quantity of dimethyl sulfoxide.

All of the experiments were repeated five to seven times. Tests on the animals were repeated five times, using five to ten animals for each test series.

Results

Phytochemical tests of the methanol extract from the flowers and leaves of *Solidago* sp. have shown the presence of a number of lipophilic flavonoids. Attempts at determining the structure of these compounds are currently in progress.

The main phenolic compound present in the extract from leaves of the species *R. chamaemorus* L. is ellagic acid, which is most probably responsible for the action of the extract from cloudberry leaves. HPLC was used to designate the quantity of ellagic acid in cloudberry leaves. The content of ellagic acid was 6.996 g/100 g of dry mass.

In addition, thin-layer chromatography was used to determine the presence of gallic acid (apart from ellagic acid) and derivatives of these two acids; the presence of a small quantity of flavones in the extracts was also confirmed.

In the leaves of an Asian species of the kudzu *P. lobata* (Willd) Ohwi, the main secondary metabolites are compounds belonging to the group of isoflavones: daidzein, genistein and formonentin. In young roots of *P. lobata*, thin-layer chromatography was used to determine the presence of an isoflavone—puerarin.

Quantitative analysis using the HPLC method for the hydrolysed and non-hydrolysed root methanol extract from *P. lobata* contained puerarin in a quantity of 5.32 mg/g dry mass and daidzein in a quantity of 5.22 mg/g dry mass in the plant extract.

The coefficients of inhibition of growth of amoebae from the genus *Acanthamoeba* sp. (IC_{50}) calculated for individual plant extracts and pure substances are presented in Table 1. It was determined that extracts from *S. virgauera*,

Table 1 IC_{50} of plant extracts for the amoebae cultures

Plant extracts	IC_{50} calculated for dry plant extracts (mg/mL)	IC_{50} calculated for active substance in plant extracts (μg/mL)
Solidago virgaurea	>0.01	nd
Solidago graminifolia	>0.05	nd
Rubus chamaemorus	>0.05	>0.350 of ellagic acid
Pueraria lobata	>0.01	>0.053 of puerarin or 0.052 of daidzein

nd not detected

Table 2 Values of the lethal dose (LD_{50}), therapeutic dose (ED_{50}) and TI for the mice

	LD_{50} (mg/g of tissue)	ED_{50} (mg/g of tissue)	TI
Solidago virgauera	3.75	0.5	7.5
Solidago graminifolia	1.75	0.5	3.5
Pueraria lobata	>2.0	nd	nd
Rubus chamaemorus	4.0	0.2	20

nd not detected

P. lobata and *R. chamaemorus* had chemotherapeutic properties in vitro in concentrations of approximately 0.01–0.05 mg extract/mL.

Table 2 presents the results of designation of therapeutic coefficients ED_{50} of tested plant extracts for laboratory mice of the BALB-c strain. The results of IC_{50} and therapeutic index (TI) research for both tested pathogenic strains of *Acanthamoeba* sp. are nearly identical and statistically significant.

Tests concerning the therapeutic action of plant extracts on the experimental infection with *Acanthamoeba* are presented in Table 3. It was determined that, following the application of the extracts, the animals survived considerably longer (2.5–3 times).

Discussion

Free-living amoebae from the genus *Acanthamoeba* are commonly occurring organisms, and they may be found in the soil, air, in each salty or drinking water reservoir and on every continent, as well as in air-conditioning units, in water mains, showers, sanitary and dental equipment, dialysers, fluids for contact lenses and infected tissue cultures (Visvesvara and Stehr-Green 1990; De Jonckheere 1991; Mergeryan 1991; Szenasi et al. 1998). The first suggestions that amoeba may cause diseases afflicting humans were made in 1958 (Marciano-Cabral and Cabral 2003). Currently, cases of GAE, *Acanthamoeba* keratitis, amoebic pneumonitis and skin inflammation are observed around the world (Fowler and Carter 1965; Callicott 1968; Jager and Stamm 1972; Willaert et al. 1976; Martinez et al. 1977; Martinez and Visvesvara 1997; Marciano-Cabral et al. 2000).

Clinical symptoms of human GAE include headaches, fever, neurological disorders, such as hallucinations, disorientation and vision disorders, personality changes and coma (Martinez and Visvesvara 1997). *Acanthamoeba* keratitis, in turn, is characterised by strong ophthalmalgia, photophobia, blue–red vision and blood extravasations. In the lungs, amoebae cause numerous inflammation foci (AP), which is accompanied by the exudation of serous fluid containing trophozoites and cysts. Skin changes are in the form of numerous, more or less extensive ulcerations. All of the infections are typically chronic.

In all cases of *Acanthamoeba* sp. invasions, chemotherapy poses a serious problem. A majority of invasions end in patient death. Only a few instances of successful chemotherapy have been noted, performed using highly toxic drugs usually used for disinfection, e.g. chlorhexidine derivatives (Kitagawa et al. 2003; Seal 2003). Effective treatment of infections of the central nervous system or eyeballs for immunocompetent persons has been recorded in the event of usage of a combined therapy, commenced at an early stage of the disease. However, in the latter stages of the disease, the majority of therapeutic agents are ineffective (Ficker et al. 1990; Dougherty et al. 1994; Horne et al. 1994; Murdoch et al. 1998).

Regardless of the wide application of therapeutic agents to combat AK, with the value thereof being unquestioned, the majority of drugs display a high toxicity for humans, causing undesirable reactions. For this reason, research is being conducted into alternative methods of treating AK, a disease which oftentimes leads to blindness and even death. Our research focused on plant extracts from *S. virgaurea*, *S. graminifolia* (goldenrod), *Rubus chamaemors* (cloudberries) and *P. lobata* (kudzu) and the potential application thereof in combating human *Acanthamoeba* sp. infections (Derda et al. 2004a, b). Drugs of natural origin have already been used to treat other parasitic diseases (Arrieta et al. 2001; Kayser et al. 2003; Said Fernández et al. 2005).

Table 1 presents results which indicate that substances present in methanol extracts from the tested plants are active with respect to both the tested pathogenic strains of amoebae from the genus *Acanthamoeba* in very low

Table 3 The survival time for mice infected with *A. castellanii* (309) following the application of the plant extracts; $n=5$ for five to ten mice

Doses of medicines	Control	0.1 mg/g of tissue	0.2 mg/g of tissue	0.5 mg/g of tissue
Survival time of infected animals				
Solidago virgauera	4±1	7±1	8±2	12±2
Solidago graminifolia	4±1	7±1	8±2	10±2
Rubus chamaemorus	4±1	5±1	10±2	10±2
Pueraria lobata	4±1	6±1	nd	nd

nd not detected

concentrations, i.e. approximately 0.01–0.05 mg extract/mL or approximately 0.35 μg/mL calculated for ellagic acid (cloudberries) and 0.53 μg/mL calculated for puerarin (kudzu), and they may be used in a combined treatment with antibiotics. The TI value is 20 for extracts from *R. chamaemors*, which is very high; for *S. virgaurea*, the TI is 7.5. Research has clearly shown that plant extracts may be successfully used both internally and externally in the event of a combined therapy against *Acanthamoeba* infections. Plant extracts administered to experimentally infected animals considerably lengthened their survival time in comparison with control animals that were not given any treatment. The control animals that were infected usually died after 4 days. The animals which received a plant extract monotherapy survived for a period 2.5–3 times longer. In the case of animals that were not infected, the therapeutic doses of drugs given did not display any toxic activity.

In the case of human infection with *Acanthamoeba* sp., a combined therapy connected with a standard antibiotic treatment would definitely be more effective than a therapy conducted using a single drug. This is so because numerous drugs have amoebistatic properties, but no amoebicidal properties. Some drugs are deadly to trophozoites, but ineffective in the case of cysts. To date, no effective drug has been found for both instances (cysts and trophozoites). Research into the efficiency of plant extracts in vivo and in vitro in an experimental *Acanthamoeba* sp. infection is being continued.

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