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Effects of medical plant extracts on the growth of the fish parasite Spironucleus vortens

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Summary

The economic importance of Spironucleus diseases led the authors to study the effect of aqueous and ethanol plant extracts on the growth of S. vortens.

In the present study 0.025 g L^{-1}, 0.05 g L^{-1} and 0.10 g L^{-1} (w/v) aquatic and ethanol extracts of tetterwort (Chelidonium majus), purple coneflower (Echinacea purpurea), garlic (Allium sativum), chestnut (Aesculus hippocastanum), horseradish (Armoracia rusticana), Bryophyllum pinnatum (Kolanchoe pinnata), oregano (Origanum vulgare), tansy (Tanacetum vulgare), thyme (Thymus vulgaris), and yarrow (Achillea millefolium) were tested against in vitro growth of Spironucleus vortens isolated from the digestive tract of discus (Symphysodon discus). The extracts of chestnut, garlic, horseradish, oregano and tansy were found to be the most effective. The 0.10 g L^{-1} extracts of these plants attained a high level of over 90% parasite growth inhibition, while their 0.025 g L^{-1} extracts, with the exception of the oregano, attained 60% parasite growth inhibition.

The results of the study confirmed that natural products are potential sources of new agents for the treatment and control of spironucleosis.

Keywords: Spironucleus vortens, fish, antiprotozoal

The diplomonads (*Diplomonadida*: *Hexamitidae*) are a group of flagellates with a double set of cellular organelles. Diplomonad flagellates, belonging to the genera *Hexamita* and *Spironucleus*, have been reported in the digestive tract of both freshwater (3, 26) and saltwater (9, 10, 30) fish. They are considered to be the pathogens causing hexamitasis and spironucleosis in fish. The flagellated protozoan from the genus *Spironucleus* (12) causes significant losses in both food and ornamental fish production (9, 24, 30). They often cause disease when the host has low resistance or is adversely affected by predisposing factors such as low oxygen content or overcrowding (15).

Spironucleus vortens commonly infects the hindgut, kidney, liver and spleen of ornamental fish – cichlids (angelfish *Pterophyllum scalare* and discus *Symphysodon discus*) and cyprinids (ide *Leuciscus idus*) (26, 31) – and is the suspected causative agent of hole-in-the-head disease, a very common affliction in ornamental cichlids (24). The disease plays an important role in cichlid cultivation and is frequently the most important profit-limiting factor in intensive aquaculture. Other parasitic members of this genus are *S. torosus*, *S. barkhanus*, *S. salmonicida* (in fish) (8, 9), *S. meleagridis* (causing diarrhoea in a wide variety of fowl) (4), and *S. muris* (commonly causing infection in mice) (1).

Metronidazole (l-(2-hydroxyethyl)-2-methyl-5-nitroimidazole) is a 5-nitroimidazole; a heterocyclic compound with a nitro group on the fifth position of an imidazole ring. It is the active compound of nitroimidazole, used in the treatment of infections induced by anaerobic bacteria and protozoa (6, 28). Metronidazole was the traditional drug of choice against spironucleosis (28), but has been banned from use in the treatment of food fish in Europe and the USA due to its potential carcinogenic properties, persistence in the environment and toxicity to aquatic organisms (13, 25, 33). The severe restrictions on the use of metronidazole highlight the need for alternative *Spironucleus* treatments in food and ornamental fish (19).

Although the literature shows that plants have huge potential in the search for new agents for treating parasite diseases, only garlic has been studied with regard to the inhibition of the growth of *Spironucleus spp*. A study by Millet et al. (19) on the influence of garlic and allium-derived products on the growth and metabolism of *S. vortens* showed that garlic and

allium-derived compounds have an inhibitory effect on *S. vortens*.

The economic importance of *Spironucleus* diseases (36) led the authors to study the effect of aqueous and ethanol plant extracts on the growth of *S. vortens*.

Material and methods

Collection of plant materials and preparation of extracts. Fresh plant material from each of the selected species was collected in 2009 in the Lublin region of eastern Poland. Plant materials were cleaned, cut into small pieces, dried (in sunlight for 7 consecutive days and then in an oven at 40°C for 24 h) and weighed. Plant sap soluble in distilled water or 40% ethanol were isolated from dried material from the following sources: the aerial part of *Achillea millefolium* (yarrow), *Chelidonium majus* (tatterworth), *Echinacea purpurea* (purple coneflower), *Kolanchoe pinnata* (= *Bryophyllum pinnatum*, air plant, life plant), *Origanum vulgare* (oregano), *Tanacetum vulgare* (tansy) and *Thymus vulgaris* (thyme); the bulb of *Allium sativum* (garlic); the seed of *Aesculus hippocastanum* (chestnut); and the root of *Armoracia rusticana* (horseradish) (Tab. 1). The dried plant materials were ground using a mortar and pestle and then soaked for 4 h in distilled water or 24 h in 40% ethanol and centrifuged at 10,000 × g for 60 min at 4°C. The ethanol was evaporated under vacuum using a rotary evaporator at 40°C. Aqueous and ethanol extracts (0.10 g L^{-1}) were bioassayed at 0.025, 0.05 and 0.10 g L^{-1}.

Metronidazole was diluted in sterile distilled water to produce twofold serial dilutions ranging from 0.0005 to 0.008 g L^{-1}.

Parasite cultivation and growth inhibition assay. *Spironucleus vortens* strain KP1, originally isolated from the digestive tract of discus (*Symphysodon discus*), was used in the experiment. The strain was grown at 28°C in a medium composed of Eagle's Minimum Essential Medium (Sigma, Poznań, Poland) supplemented with 10% calf serum (Biomed, Lublin, Poland) and 4% Keister's modified bile-supplemented TYI-S-33 medium (11).

The experiment was carried out using Eppendorf tubes containing 10^3 protozoa/ml. The extracts to be tested were added to the cultures 3 h after seeding (0 h). Viable protozoa were assessed at 72 h after incubation with the extracts. Protozoa were harvested from the culture and trophozoites were enumerated by manual counting with a haemocytometer.

Growth rate (GR) was defined as the difference between the number of living protozoa counted at 0 h and after 72 h. The percentage of growth inhibition (GI) was calculated using the following formula (20):

$$\%GI = \left(1 - \frac{GR_{Extract}}{GR_{Control}}\right) \times 100$$

The experiments were performed in triplicate and repeated twice.

Results and discussion

In this study we investigated the bioactivity of ten naturally growing plants (Tab. 1). Among the plant species evaluated, *A. sativum*, *A. hippocastanum*, *A. rusticana*, *O. vulgare* and *T. vulgare* presented the best results in terms of anti-*Spironucleus* activity. The aqueous extracts displayed 100%, 80.39%, 100%, 100% and 99.57% growth inhibition, respectively, of *S. vortens* KP1 at the highest concentration tested, 0.10 g L^{-1}, while the ethanol extracts displayed 100%, 99.57%, 99.15%, 100% and 97.01% growth inhibition, respectively (Tab. 2).

Tab. 1. Plants selected for *in vitro* investigation and parts of plants studied

Plant species	Family	Plant part
Yarrow (*Achillea millefolium*)	*Asteraceae*	aerial part
Garlic (*Allium sativum*)	*Amaryllidaceae*	bulb
Chestnut (*Aesculus hippocastanum*)	*Sapindales*	seed
Horseradish (*Armoracia rusticana*)	*Brassicaceae*	root
Tetterwort (*Chelidonium majus*)	*Papaveraceae*	aerial part
Purple coneflower (*Echinacea purpurea*)	*Asteraceae*	aerial part
Air plant (*Kolanchoe pinnata*)	*Crassulaceae*	aerial part
Oregano (*Origanum vulgare*)	*Lamiaceae*	aerial part
Tansy (*Tanacetum vulgare*)	*Asteraceae*	aerial part
Thyme (*Thymus vulgaris*)	*Lamiaceae*	aerial part

Tab. 2. Anti-*S. vortens* activity of medical herb extracts (g L^{-1}) and metronidazole (g L^{-1})

Plant species	Extract	Growth inhibition (%) Mean ± SD		
		0.025 g L^{-1}	0.05 g L^{-1}	0.10 g L^{-1}
A. hippocastanum	Aqueous	47.55 ± 6.99	55.36 ± 5.92	80.39 ± 5.32
	Ethanol	76.18 ± 1.65	90.24 ± 2.24	99.57 ± 0.42
A. millefolium	Aqueous	7.71 ± 4.26	12.06 ± 2.06	47.39 ± 8.52
	Ethanol	6.55 ± 1.44	54.86 ± 6.47	85.26 ± 4.36
A. rusticana	Aqueous	58.96 ± 5.47	98.78 ± 1.17	100.00 ± 0.00
	Ethanol	61.24 ± 2.87	93.62 ± 6.09	99.15 ± 0.82
A. sativum	Aqueous	70.30 ± 5.07	99.21 ± 1.37	100.00 ± 0.00
	Ethanol	83.73 ± 3.23	96.34 ± 3.50	100.00 ± 0.00
C. majus	Aqueous	32.36 ± 3.91	48.03 ± 9.85	75.05 ± 6.29
	Ethanol	51.37 ± 5.87	60.58 ± 3.33	78.93 ± 4.58
E. purpurea	Aqueous	–7.93 ± 6.92	–7.17 ± 6.54	–1.96 ± 9.46
	Ethanol	8.90 ± 2.62	19.77 ± 2.77	47.69 ± 4.60
K. pinnata	Aqueous	11.08 ± 3.61	15.22 ± 5.65	30.93 ± 6.82
	Ethanol	4.31 ± 4.16	21.15 ± 3.08	22.78 ± 6.78
O. vulgare	Aqueous	6.49 ± 4.25	31.23 ± 16.00	100.00 ± 0.00
	Ethanol	26.98 ± 2.67	82.08 ± 3.82	100.00 ± 0.00
T. vulgare	Aqueous	67.30 ± 6.46	79.08 ± 3.08	99.57 ± 0.43
	Ethanol	64.73 ± 3.34	83.76 ± 3.44	97.01 ± 3.46
T. vulgaris	Aqueous	2.44 ± 5.73	6.69 ± 4.45	63.84 ± 15.74
	Ethanol	–5.25 ± 4.36	24.47 ± 7.34	71.15 ± 8.25
Metronidazole		0.0005 g L^{-1}	0.001 g L^{-1}	≥ 0.002 g L^{-1}
		82.50 ± 6.70	98.75 ± 2.50	100.00 ± 0.00

S. vortens is a parasite of considerable economic and veterinary importance in the aquaculture industry. Numerous compounds have been used to treat spironucleosis in farmed fish. Natural compounds produced by plant secondary metabolism are potentially a very important source of new types of drugs. Although numerous studies have shown that natural products can be an excellent source of new agents for parasitic disease control, there have only been a few studies on *in vitro* testing of plant material against *Spironucleus* spp. (19, 35).

A. sativum and allium-derived compounds have exhibited antimicrobial activity against many parasitic protists, including *Trypanosoma*, *Giardia*, *Entamoeba* (7, 17, 23) and *Spironucleus* (19, 35). In our study, crude extract of garlic exhibited high anti-*Spironucleus* activity. The aqueous and ethanol extracts of *A. sativum* exhibited 99.21% and 96.34% anti-*Spironucleus* activity, respectively, at a concentration of 0.05 g L^{-1} (Tab. 2). This observation supports earlier work by Millet et al. (19) in which allicin, dithiins and ajoene inhibited the growth of *S. vortens* with MIC values much higher than those reported for most bacteria, fungi and protozoa. Proteome analysis indicates that garlic derivatives act by diverse mechanisms independently of those attributable to metronidazole, and also disturb redox balance (35).

The usefulness of *A. hippocastanum* and *A. rusticana* in the treatment of protozoal infections has not previously been investigated. To the author's knowledge, this is the first report on the antiprotozoal activity of *A. hippocastanum*. The aqueous and ethanol extracts of *A. hippocastanum* exhibited 55.36% and 90.24% anti-*Spironucleus* activity, respectively, at a concentration of 55 mg/ml. The aqueous and ethanol extracts of *A. rusticana* exhibited 100% and 99.15% anti-*Spironucleus* activity, respectively, at 0.10 g L^{-1} (Tab. 2).

Previous studies on the activity of extracts of *A. illefolium*, *C. majus*, *E. purpurea*, *K. pinnata*, *O. vulgare*, *T. vulgare*, and *T. vulgaris* against protozoal infections have yielded positive results (2, 16, 21, 22, 29, 37), but their usefulness in the treatment of *Spironucleus* has not previously been investigated.

In this study, the positive control against *S. vortens* was metronidazole, which inhibited the parasite's growth at concentrations of 0.0025 g L^{-1} or higher (Tab. 2). Our observations are consistent with previous studies (28). Metronidazole is the most widely prescribed drug in the treatment of anaerobic protozoa (6, 28). The drug enters the cell through passive diffusion, and there a nitro group is subsequently reduced to reactive cytotoxic nitro radicals by reduced ferrodoxin or flavodoxin. This nitro radical is hypothesized to bind transiently to DNA, disrupting or breaking the strands and leading to cell death (5, 14). Treatment with metronidazole is usually highly effective (28). However, metronidazole resistance is well documented in various protozoan species (27, 34), including *Spironucleus sp*. (28). The recommended dose is 2-5 g per 1 kg feed for food fish (32) and 10 mg per 1 g fish food for ornamental fish (18).

In summary, we have described the antiprotozoal properties of aqueous and alcoholic fractions of *A. hippocastanum*, *A. sativum*, *A. rusticana*, *O. vulgare* and *T. vulgare* against *S. vortens* and concluded that extracts of these plants are suitable candidates for antiprotozoal drug discovery.

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