Toxic action of substances from male fern Dryopteris filix-mas (L.) Schott (1834) on free-living soil nematode *Caenorhabditis elegans* Maupas (1900)

Anastasia Egorova[1], *Alsu* Gatiyatullina[1], *Dmitriy* Terenzhev[2], *Timur* Belov[2], and *Tatiana* Kalinnikova[1,*]

[1]Research Institute for Problems of Ecology and Mineral Wealth Use of Tatarstan Academy of Sciences, 420087 Kazan, Russian Federation
[2]Federal State Budgetary Institution of Science, Federal Research Center, Kazan Scientific Center of Russian Academy of Sciences, 420111 Kazan, Russian Federation

Abstract. The study of biological activity of extracts from roots and rhizomes of male fern *Dryopteris filix-mas* was carried out in experiments with soil nematode *Caenorhabditis elegans*. The toxicity of extracts of *D. filix-mas* roots and rhizomes obtained by different methods varied over a wide range. Crude extract of male fern roots and rhizomes in concentration range 62.5–250 µg/ml had a weak toxic action on *C. elegans* organism by inducing death of 26.0–43.3% nematodes. The toxicity of relatively high concentrations (500 and 250 µg/ml) of water-ethanolic extracts might be compared with such of crude extract in concentrations 62.5–250 µg/ml. Biological activity of filicinic acids ethers was found very high: concentrations of crude extract of *D. filix-mas* roots and rhizomes and concentrations of filicinic acids ethers with similar toxicity for *C. elegans* organism were 40-fold different. Substances isolated from *D. filix-mas* roots and rhizomes have nematicidal activity and may be used for development of drugs to treat helminthiasis of humans and animals.

1 Introduction

Helminthiases are one of the most common diseases of livestock and domestic pets. Animals infected by helminths decline their producing capacity. Helminths infections make animals more vulnerable to other diseases owing to immunity impairment. Helminthiases may cause lesion of eyes, gastrointestinal tract, cardiovascular and respiratory systems of humans. Not less than two billion people are affected by different helminthiases, and most of these people live in tropical and subtropical regions [1]. Helminthiases are serious problem not only for medicine, but also for veterinary medicine. The weight of animals infected by helminths is reduced for 3–8%, and their death increases by about 28% [2].

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* Corresponding author: tbkalinnikova@gmail.com

Since ancient times people used medicinal plants to treatment diseases caused by parasites. After appearance of safety and effective synthetic anthelmintics, medicinal herbs lost their actuality for medicine and veterinary [3]. Nevertheless, the World Health Organization has recently estimated that 80% of the population of development countries uses plant drugs for health care needs. Modern pharmacopoeia contains in the order of 25% drugs derived from plants and many other drugs are synthetic analogues of compounds isolated from plants [2, 4]. Today we can say with confidence that phythotherapy entered its Renaissance. This reveals in increasing interest to ethnobotany and in use of biologically active compounds extracting from plants as alternative for synthetic chemical drugs due to their high effectiveness, economic profitability, ecological and biological safety [5–6].

In veterinary medicine the interest in use of plants for helminthiases treatment is defined by the fact that none of synthetic drug applicable today meets the ideal requirements of anthelmintics, such as broad spectrum of activity, single dose cure, free from side effects and cost-effectiveness [2]. One also should not forget about the origin of drug resistance of parasites because of regular use of synthetic anthelmintics. For example, parasitic nematode *Haemonchus contortus*, infesting ruminants, now is resistant to all groups of anthelmintic drugs [7]. Medicinal plants used for treatment of parasitic diseases in veterinary, have very little side effects and do not increase the resistance to synthetic anthelmintic drugs. The interest to looking for new anthelmintic drugs based on plant preparation is recently increased not only in connection with rise of drug resistance of parasites, but also in connection with the possibility of synthetic anthelmintics entrance through food chain into human foodstuffs [4, 8].

Folk medicine traditionally uses spermaphytes [9–10]. Higher sporophytes are used in folk medicine and ethnoveterinary more rarely, although medicinal qualities of ferns were known long ago [7, 11]. In the work of Rajesh et al. [7] phytochemical analysis of six fern species growing in India was performed, and their effectiveness against *Haemonchus contortus* in ewes was shown. The database of Ukrainian ferns was built up by Ukrainian researchers. Among 63 fern species included in this database, 39 contain biologically active compounds, which may be used in medicine and as food additives [11].

Medicinal properies of male fern *Dryopteris filix-mas* are described in research literature most often. The main active compound of male fern is crude or "raw" filicin, which is the mixture of biologically active compounds, main of them are filixic and filixidinic acids, isomers of filixinic acid, flavaspidic acid, kaempferol, kaempferol-3-o-glucoside, rutin, albaspidin and aspidinol [12].

Today the therapeutic action of anthelmintic drugs is studied in experiments in vivo with animals infected by helmints. The effectiveness of drug is estimated by helminths' eggs content and died helminths in animals' faeces [8]. Nematicidal activity in vitro is studied more rarely because the breeding of parasitic worms in laboratory is not always possible. The convenient model organism for investigation anthelmintic activity of synthetic drugs is free-living soil nematode *Caenorhabditis elegans*, because its body plan, physiology and neurochemistry are very similar with such of parasitic nematodes [1].

The aim of this work was investigation of anthelmintic action of male fern *D. filix-mas* extract and its separate components in experiments with *C. elegans*.

2 Materials and methods

2.1 Preparation of crude extract from roots and rhizomes of *Dryopteris filix-mas*

Roots and rhizomes of fern were washed away from soil and then dried during 24 hours in drying oven with forced ventilation. Dried raw material was crushed to parts size 0.1–3 mm. Extraction was performed with chloroform by Soxhlet, where three filter packs each containing 7 g of pounded material were placed in a thimble. In total five extraction cycles were performed to full decoloration of outgoing chlorophorm. Ammonium solution was added to extract till pH 8.9, and then extract was shaken for 30 minutes with ELMI S-3M shaker. Then the mixture was filtered and transferred to a separatory funnel till close-cut separation of phases. The extract of roots and rhizomes divided to three phases. Aqueous layer containing fraction of ammonium salts of filicinic acids was separated and concentrated with rotary evaporator. Middle layer containing filicinic acids ethers was dried and re-extracted with 50% ethanol.

2.2 Preparation of aqueous-ethanolic extracts of roots and rhizomes of *Dryopteris filix-mas*

Roots and rhizomes of fern were washed away from soil and then dried during 12 hours in drying oven at temperature of 45°C. Dried raw material was crushed to parts size 0.1–0.5 mm. Then triple maceration of roots and rhizomes (15 g each) in eightfold volume (120 ml) of solvent was performed with chloroform, each for 1.5 hours at temperature of 50°C. After third stage the rough filtration through lavsan filters was carried out followed by precipitation of suspended particles for 15 min at 4500 rpm (centrifuge VS-1536E) and final filtration through SF paper (slow filtration). Obtained extract was separated into two equal volume parts and dried under reduced pressure in vacuum rotary evaporator IR-1LT (15 mmHg, 25°C) to full remove of extragent [13, 14].

To prepare **sample 1**, the first part of dried solid residue was redissolved with 7% ethanol. To prepare **sample 2**, 7% ethanol was added to another part of dried extract, and hydrochloric acid was added to pH 3.0. The extract was warmed for 1.5 hours at temperature of 70℃ for ethers hydrolysis and isolation of free filicinic acids. For neutralization of residual amounts of hydrochloric acid sodium hydrocarbonate was used [15].

Table 1. Phytochemical characteristics of extracts of *Dryopteris filix-mas* roots and rhizomes

	pH	Total phenolic number, mg GAE/g of raw material	Total amount of phlavonoids, mg Rut/g of raw material	Kaempferol-3-O-glucoside, mg/g of raw material	Filixic acid, mg/g of raw material
Roots and rhizomes extract (salt phase)	7,93	7,32	3,52	0,012	0,023
Roots and rhizomes extract (ether phase)	5,73	112,25	75,5	7,52	1,29
Sample 1	6.35	112.5	75.3	0,35	0,18
Sample 2	6.76	129.7	39.7	0,021	0,035

The total content of phenolic compounds was estimated in aqueous-ethanolic extracts of *D. filix-mas* by Folin-Ciocalteu method. Total content of phenolic compounds was expressed in milligrams of gallic acid equivalent (GAE) in 1 g of material [16]. The content of flavonoids was determined according method described by M. Stanković [17]. The

content of flavonoids was expressed in rutin equivalent (Rut) in 1 g of raw material. The content of kaempferol-3-O-glucoside was determined by HPLC method using standard [15]. The amount of filixic acid was proved by combine method with use of HPLC, NMR C^{13} and H^1 [18]. Phytochemical characteristics of obtained extracts are shown in Table 1.

2.3 In vitro assays of toxicity of *Dryopteris filix-mas* roots and rhizomes extracts

Experiments were performed with young adult hermaphrodites *C. elegans* of wild type strain N2 received from Caenorhabditis Genetic Center. Nematodes were grown at 22°C in Petri dishes with standard Nematode Growth Media seeded with *E. coli* OP50 as food resource [19]. All experiments were carried out in M9 buffer (3 g/l KH_2PO_4, 6 g/l Na_2HPO_4, 5 g/l NaCl, 1 мM $MgSO_4$) [19]. After washing away from growth media, bacteria and exomethabolites [20] nematodes were transferred into glass centrifuge tubes 10 ml in volume (50 worms in each tube). After worms' settling on tubes' bottom supernatant was removed, extracts from *D. filix-mas* were added, and M9 buffer was added to final volume of 1 ml. The relevant amount of ethanol was added into control tube. Into all tubes (both control and experimental) 1% on dimethyl sulfoxide was added. Tubes with nematodes were incubated for 24 hours at temperature of 22°C. The number of dead nematodes was used as index of toxic action of *D. filix-mas* extracts. All experiments were performed five times.

3 Results and discussion

Soil nematode *C. elegans* is very sensitive to most modern synthetic drugs using for treatment of human and animals' helminthiases. The basis for study of anthelmintic activity of biologically active compounds is their ability to alter growth, development, metabolism and/or behavior of nematodes [1]. We have chosen the nematode death after 24-hours incubation with roots and rhizomes extracts as criteria for toxicity of *D. filix-mas* for *C. elegans*.

Table 2. Toxic action of crude extract *Dryopteris filix-mas* roots and rhizomes on *Caenorhabditis elegans*

The percentage of dead nematodes, %			
Concentration of crude extract of *Dryopteris filix-mas* roots and rhizomes, µg/ml			
0	250	125	62.5
5.3±1.8	43.3±4.0	34.0±3.9	26.0±3.6

Comment: nematodes were exposed to toxicants for 24 hours.

Table 3. Toxic action of filicinic acids ethers on *Caenorhabditis elegans*

The percentage of dead nematodes, %								
Concentration of filicinic acids ethers, µg/ml								
0	100	50	25	12.5	6.25	3.12	1.56	0.78
5.3±1.8	93.3±2.0	92.0±2.2	89.3±2.5	73.3±3.6	61.3±3.9	32.0±4.7	28.7±4.5	20.0±4.0

Comment: nematodes were exposed to toxicants for 24 hours.

Table 4. Toxic action of aqueous-ethanolic extracts of roots and rhizomes
of Dryopteris filix-mas on Caenorhabditis elegans

The percentage of dead nematodes, %				
Concentration of **sample 1**, µg/ml				
0	1000	500	250	125
0	100	85.2±2.2	35.6±3.0	1.6±1.0
Concentration of **sample 2**, µg/ml				
0	500	250	125	62.5
0	88.8±2.0	48.8±3.2	9.6±1.9	1.2±0.9

Comment: nematodes were exposed to toxicants for 24 hours.

In our experiments crude extract of male fern roots and rhizomes in concentrations range 62.5–250 µg/ml had slight toxic effect on *C. elegans* organism by inducing the death of 26.0–43.3% of nematodes (Table 2). Filicinic acids ethers were essentially more toxic for *C. elegans*, inducing the death of more than 90% of worms in concentrations 100 and 50 µg/ml (Table 3). Crude extract of roots and rhizomes of male fern caused the death of 34% of nematodes after 24-hours exposition in concentration 125 µg/ml, whereas filicinic acids ethers induced death of similar number of nematodes (32%) in concentration as small as 3.12 µg/ml. Similar toxic action on nematodes organisms demonstrated crude fern extract in concentration 62.5 µg/ml and filicinic acids ethers in concentration 1.56 µg/ml inducing the death of 26 and 28.7% of nematodes respectively. This is in accordance with high content of active compounds – filixic acid and kaempferol-3-O-glucoside in comparison with other extracts (Table 1, 3).

Aqueous-ethanolic extracts of ground parts of *D. filix-mas* were the least toxic for *C. elegans* (Table 4). **Samples 1** and **2** in concentration 500 µg/ml caused the death of 85.2 and 88.8% of nematodes respectively. In concentration 250 µg/ml these extracts caused the death of 35.6% of nematodes (**sample 1**) and 48.8% of nematodes (**sample 2**) which may be compared with toxicity of crude extract (Table 2, 4).

The nematodes death under the action of synthetic nematicides may be caused by different reasons depending on the nature of compound, target and mechanism of its action. Mechanisms of toxic action of most synthetic nematicides are well studied. It is known that nematicide action of benzimidazoles (mebendazole, albendazole, etc.) is determined by disruption of β-tubulin synthesis and alteration of carbohydrate, lipidic and energy metabolism [1]. Macrocyclic lactones (ivermectin, avermectin) elicit a potent and persistent paralysis of nematode pharyngeal and body wall musculature provoking the death of organism. The targets for macrocyclic lactones action are glutamate-gated chloride channels. The target for most anthelmintic drugs action is nematodes nervous system, though mechanisms of toxic action may be different. Acethylcholine esterase inhibitors both organophosphorous (carbophos, dichlorvos, etc.) and carbamate (aldicarb) increase acethylcholine concentration in intercellular space. This leads to hyperactivation of acethylcholine receptors which cause muscles hypercontraction followed by paralysis and death of nematodes. Imidazothiazoles and tetrahydropyrimidines (levamisole, pyrantel, etc.) are agonists of nicotinic receptors of acethylcholine. The mechanism of their toxic action consists in stimulation of nicotinic cholinoreceptors, which leads to muscle paralysis and nematode death. Spiroindoles (derquantel and paraherquamide), in contrast, are antagonists of nicotinic cholinoreceptors, and mechanism of their toxic action is disruption of cholinergic synaptic transmission [1].

At present we have no sufficient scientific basis for use of plant-derived substances for treatment helminthiases of humans and animals. Plant extracts are complex multicomponent mixtures of compounds with different biological activity and different mechanisms of action. Many plants contain compounds affecting cholinergic systems:

acethylcholine esterase inhibitors, agonists and antagonists of acethylcholine receptors [9, 21]. Tansy *Tanacetum vulgare*, wormwood *Artemisia absintium* and some other plants using for helminthiases treatment contain thujone – antagonist of one of subtypes of γ-aminobutiric acid receptors [9, 22]. Valerenic acid containing in ground parts of *Valeriana officinalis* is agonist of serotonin receptors with high affinity to 5-HT$_{5A}$ receptor and low affinity to 5-HT$_{2B}$ receptor and serotonin transporter [23]. Serotonin, in turn, increases sensitivity of nicotinic cholinoreceptors to their agonists [24]. The same plant may content compounds affecting different molecular targets in animal organism. So, it is very difficult to predict the final result of effects of multicomponent mixture.

In our experiments with *C. elegans* toxicity of extracts of *D. filix-mas* obtained by different methods varied in a wide range. Toxicity of relatively high doses (500 and 250 µg/ml) of aqueous-ethanolic extracts might be compared with toxicity of crude extract in concentrations 62.5–250 µg/ml (Table 2, 4). Biological activity of filicinic acids ethers was very high: concentrations of filicinic acids ethers were 40-fold higher than concentrations of crude extract of *D. filix-mas* roots and rhizomes with similar action on *C. elegans* organism (Table 2, 3).

4 Conclusions

1. Extracts of male fern *Dryopteris filix-mas* caused dose-dependent death of nematode *Caenorhabditis elegans*.

2. Toxicity of extracts of male fern ground parts depended on the method of extraction. Biological activity of crude extract was similar with biological activity of aqueous-ethanolic extracts.

3. Filicinic acids ethers were very toxic for *C. elegans*. Concentrations of filicinic acids ethers were 40-fold higher than concentrations of crude extract of *D. filix-mas* roots and rhizomes with similar action on *C. elegans* organism.

4. Preparations derived from *D. filix-mas* roots and rhizomes possess nematicidal activity and can be used for preparation of medicaments for treatment helminthiases of humans and animals.

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