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RESEARCH ARTICLE

Effects of a *Cyclachaena Xanthiifolia* Extract on *Trematodes Opisthorchis Felineus*: An in Vitro Study

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Abstract: *Cyclachaena xanthiifolia* is a phytochemically rich invasive weed with documented allelopathic properties, making it a candidate source of bioactive compounds with potential medicinal applications. This study investigated the anthelmintic activity of a crude aqueous-ethanolic extract of *C. xanthiifolia* (CxEt), prepared from equal proportions of leaves and seeds, against the trematode *Opisthorchis felineus in vitro*. The effects of CxEt on the motility and mortality of newly excysted metacercariae (NEMs) and adult flukes (maritae) of *O. felineus* were assessed across a concentration range of 0.1-1000.0 µg/mL. Secondary metabolite content was quantified, antiradical activity was determined by the DPPH assay, and cytotoxicity was evaluated by the MTT assay using cultured HFF10 cells. CxEt demonstrated strong anthelmintic activity: half-maximal inhibitory concentrations (IC50) for motility reduction were 13.4 µg/mL for NEMs and 10.9 µg/mL for maritae. Complete lethality at 24 h was achieved at 800 µg/mL for NEMs and 1000 µg/mL for maritae, with lethal effects observed at lower concentrations (400-600 µg/mL) over 3-4 days of continued observation. Cytotoxicity assessment yielded an IC50 of 168 µg/mL after 72 h of exposure, indicating a favorable selectivity profile relative to the anthelmintic IC50 values. CxEt also demonstrated moderate antiradical activity (DPPH IC50 580 µg/mL). Elevated concentrations of triterpene saponins (4.03%) and phenolic compounds, including tannins and hydroxycinnamic acids (8.20 mg GAE/g) were identified as likely contributors to the observed bioactivity. These findings establish *C. xanthiifolia* as a promising source of opisthorchicidal compounds and support its further investigation as a plant-derived anthelmintic agent.

Keywords: *Cyclachaena xanthiifolia*, *Opisthorchis felineus*, Anthelmintic activity, Opisthorchiasis, Trematode, Phytochemical analysis, DPPH assay, MTT cytotoxicity

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Introduction

Trematodes Opisthorchis felineus, *Opisthorchis viverrini*, and *Clonorchis sinensis* are representatives of the Opisthorchiidae family; they are parasites of humans and animals and are widespread in vast regions of Europe and Asia [1]. There is a known influence of opisthorchiids on the development of life-threatening pathologies in humans. Many studies

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have confirmed the association between opisthorchiasis and cholangiocarcinoma, and this connection is a global healthcare problem [2-4]. Scientists from Western Siberia have demonstrated a close relation between *O. felineus* infestation and cases of cholangiocarcinoma [5]. *Opisthorchis felineus* is widespread in Europe and Siberia (especially the Ob-Irtysh river basin) with an infection prevalence affecting more than 1.2 million people [6].

To date, praziquantel remains the main effective drug of choice for the treatment of opisthorchiasis [7]. Nevertheless, its treatment effectiveness is not always 100% because this compound is quickly eliminated from the human body and has a number of adverse effects [8]. In addition, experiments with reinfection of rats with the trematode *Clonorchis sinensis* have shown that praziquantel treatment may induce resistance to secondary infection. To alleviate side effects of praziquantel in the host, it is combined with vegetable substances able to mitigate the adverse action of the drug. For instance, praziquantel is applied in combination with curcumin, which itself can exert anti-inflammatory, antioxidant, and antitumor actions [9]. The combined use prevents the development of periductal fibrosis in the hamster liver in *O. viverrini*-induced opisthorchiasis, thereby improving the morphology of bile ducts. Therefore, screening of bioactive substances of natural origin is still relevant and is a source of promising anthelmintics [10]. The main reason is that the native chemical structure of such compounds, owing to the similarity of metabolism between plant and animal cells, can ensure effective pharmacological interaction between cellular components and these compounds, along with the low probability of adverse reactions in the human body.

Cyclachaena xanthiifolia Nutt. (syn. *Iva xanthiifolia*) is a herbaceous plant of the family Asteraceae Dum. It is regarded as an actively spreading weed; this species is recognized as invasive [11]. *Cyclachaena xanthiifolia* quickly invades new phytocoenoses and often displaces native plant species [11-13]. The successful spread of this species is explained by its biological features, in particular, the phytochemical composition of organs and tissues. For example, phytoanticipins (phytoprotectors) make plants resistant to infections and phytophages, whereas allelopathic substances help to reduce the viability of competing plants [13,14]. Yao et al. [14] identified 70 compounds, including 2-camphor, borneol, isovanillin, 2-methylallyl phenol, dibutyl phthalate, 1-caryophyllene, ambrosin, and coronopilin. Some of these compounds are known to have antiparasitic and antifungal properties. For example, ambrosin is effective against seven strains of *Trypanosoma cruzi* (the etiological agent of Chagas disease) [15], and lactone coronopilin can inhibit the growth of fungal pathogens [16]. Considering the pharmacological properties in plants of the entire Asteraceae family caused by such substances as terpenoids, alkaloids, phenolic compounds, and polyacetylenes [17], it can be hypothesized that a representative of this family, *C. xanthiifolia*, has anthelmintic activity. Additionally, a moderate antinematode efficacy of a 3% aqueous extract of *C. xanthiifolia* against larval forms of *Strongyloides papillosus* has been previously shown [18].

Nonetheless, no influence of *C. xanthiifolia* extracts on other taxa of helminths is known. In particular, an effect of this plant on the liver fluke *Opisthorchis felineus* has not been reported. It is obvious that any study on antiparasitic substances should begin with in vitro experiments prior to attempts to determine their effects in vivo [19]. Therefore, the purpose of our work was to investigate in vitro the anthelmintic potential of a crude aqueous-ethanolic extract of *C. xanthiifolia* (CxEt) at two developmental stages of the trematode *O. felineus*: in Newly Excysted Metacercariae (NEMs) and in adult flukes (maritae). Furthermore, phytochemical and cytotoxic properties of CxEt needed to be determined for subsequent in vivo studies.

Materials and Methods

Ethical Approval

All animal manipulations were carried out in accordance with the guidelines of the International Organization of Medical Sciences (CIOMS) on the ethics of clinical research and in accordance with the decision of the Committee on Bioethics of IC&G SB RAS (Protocol No. 39 dated September 27, 2017).

Sampling of Plant Materials and Preparation of the Extract

Raw material of *C. xanthiifolia* was collected in September 2022 in the territory of Pavlodar city (Republic of Kazakhstan). Species affiliation of the plant was determined by Dr. M.N. Lomonosova (Central Siberian Botanical Garden, SB RAS, Novosibirsk, Russian Federation) (*C. xanthiifolia* collection, collection No.: NS0059244). Plants without signs of damage were dried at room temperature and were rid of large stems; seeds and leaves were crushed in equal proportions. CxEt was prepared in a water bath at 95°C by threefold extraction with 10 mL of solvent per 1 g of plant material. Specifically, 22 g of dried crushed *C. xanthiifolia* were incubated in 220 mL of 50% aqueous ethanol. After extraction, CxEt was concentrated in a

vacuum centrifugal concentrator (Eppendorf Concentrator plus, Eppendorf, Germany) at 40°C to dryness and stored at −20°C.

Extraction of O. felineus Metacercariae

The *O. felineus* metacercariae were isolated from ide (*Leuciscus idus*), which were fished from the Ob River near Novosibirsk city, according to the method described earlier [20]. The larvae were repeatedly washed with sterile saline solution. Then, under the action of a 0.06% trypsin solution in a CO_2 incubator (5% CO_2, 37°C) (Thermo Hera cell 150), the process of metacercaria release from cysts was monitored for 15-20 min.

NEMs were washed with RPMI 1640 culture medium supplemented with L-glutamine (Sigma-Aldrich, USA), 1% glucose, 100 μg/mL streptomycin, 100 U/mL penicillin, and 0.25 μg/mL amphotericin B (Gibco, USA). NEMs were placed (50 ± 5 individuals per well) in a 12-well culture dish (NEST, China).

Extraction of O. felineus Maritae

Ten Syrian hamster (*Mesocricetus auratus*) males were received from the Animal Laboratory (IC&G SB RAS). The animals were intragastrically infected with *O. felineus* metacercariae (100 larvae per hamster) using special probes (Braintree Scientific, Inc., Braintree, MA). Hamsters were euthanized after three months, and *O. felineus* maritae were isolated from the liver ducts [21]. Adult flukes, after washing in sterile saline solution, were placed in culture medium for 24 h at 37°C. After that, maritae were distributed over the wells of a 12-well culture plate (7-8 individuals per well) with 1 mL of RPMI 1640 medium. Two wells were used for each concentration of CxEt.

Experimental Design

The stock solution of CxEt was prepared with sterile distilled water so that final concentrations of the extract in RPMI 1640 incubation solutions were 0.1, 1, 10, 100, 200, 400, 600, 800, and 1000 μg/mL. Each concentration was tested in two replicates. In negative control wells, NEMs and maritae were added with sterile vehicle equivalent to the volume of the stock treatment solution. Trematodes were kept in a humidified atmosphere with 5% CO_2 at 37°C.

The motility of NEMs was assessed using a microscope (Axiovert 40CFL, Carl Zeiss, Jena, Germany) at 3, 24, 48, 72, and 96 h after the addition of CxEt or sterile H_2O, and the motility of adult flukes was assessed using a stereomicroscope (ZEISS Stemi 305) at 3, 24, 48, and 72 h.

The movements of trematodes were estimated on a 4-point scale [20,22]. Each of two independent observers visually recorded the pattern and intensity of trematode movements in each well for 3 min. The data were pooled and averaged. Further analysis of motility data was performed using non-parametric Kruskal-Wallis one-way analysis of variance (ANOVA) in Statistica 6.0, followed by post hoc pairwise comparisons using the Mann-Whitney U test. All values are presented as mean ± SEM.

The half-maximal inhibitory concentration of the extract (IC_{50}) was calculated using CompuSyn 1.0 software (CompuSyn, Inc.) [23]. The relative mobility index (RM) was calculated using equations as described in previous studies [20,24]. A fluke survival test after administration of CxEt was performed for four days (NEMs) and three days for adult flukes. Viability of trematodes was assessed by staining with 0.1% eosin [20,25].

Cyclachaena xanthiifolia Secondary Metabolite Levels and Determination of Antiradical Activity by DPPH Assay

Secondary metabolites of *C. xanthiifolia* were quantified using spectrophotometric methods described in [26,27]. The antioxidant activity of CxEt was determined by the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay [26].

The Cytotoxicity Assay of the Extract from C. Xanthiifolia

In order to confirm the absence of high toxicity of CxEt to the host of *O. felineus* helminths in expected in vivo experiments, cytotoxicity was determined in a cell culture model using normal human fibroblasts. The HFF-10 cell line (human foreskin fibroblasts) was grown in F12/DMEM medium (Sigma-Aldrich) supplemented with 10% fetal bovine serum under standard conditions (humidified atmosphere with 5% CO_2 at 37°C). One day before testing, cells were seeded in a 96-well plate at 30,000 cells per well. Treatment with CxEt at concentrations of 0.1, 1, 10, 100, 1000, and 10,000 μg/mL lasted for 72 h, with four biological replicates for each concentration. Cell viability was determined using the MTT assay according to a standard

protocol [28]. Optical density (OD) was measured at λ = 580 nm using a VICTOR X microtiter plate reader (PerkinElmer). Optical density values were converted to inhibition percentage according to the equation:

$$I\% = 100 - (\text{OD treated} - \text{OD blank})/(\text{OD PBS} - \text{OD blank}) \times 100\%$$

Dose-response curves were evaluated under a four-parameter nonlinear regression model in WorkOut 2.5 software. IC_{50}, IC_{95}, and IC_5 were deduced from the dose-response curves.

Results

Effects of CxEt on NEMs of O. felineus

All NEMs that were kept in RPMI 1640 without CxEt (negative control) maintained high motility throughout the experiment (Tables 1 and 2, Fig. 1a). The motility of NEMs in the control group was always higher than in other groups at any substance concentration and at all time points, with the exception of the dose 0.1 µg/mL. At this dose, NEMs motility after 24 h did not differ from the control value according to the Mann-Whitney U test. On day 4 of observation, the motility of control NEMs decreased by only 13%, and dead NEMs constituted only 2% (Table 1).

After 3 h of exposure to CxEt, a slight decrease in NEMs motility was observed at high doses, but all 1485 individuals were alive. Effects on motility were recorded 24 h after exposure to CxEt at concentrations of 400 µg/mL or more, and immotility remained throughout the subsequent observation period (Table 1).

After 24 h of exposure to 0.1-100 µg/mL CxEt, mortality of NEMs was zero (Table 2). At 200 µg/mL CxEt, within 24 h, only 2% of NEMs died, but the remaining live individuals lost the capacity to actively move (RM = 41). At CxEt doses of 400-600 µg/mL, high mortality of NEMs was observed, and at 800-1000 µg/mL, mortality reached 100%. Dead worms showed noticeable morphological changes, including a swollen body, blurred structure of internal organs, and an enlarged excretory bladder (Fig. 1e, f). Such alterations were most pronounced at 1000 µg/mL CxEt. After longer observation periods (48 and 72 h), a dose-dependent decrease in motility of NEMs was also observed, starting at lower doses of CxEt (100-200 µg/mL) (Table 2). During these periods, high doses of CxEt (400-600 µg/mL) caused 100% mortality of NEMs. After 96 h of observation at low doses of CxEt (0.1-10 µg/mL), motility of NEMs gradually diminished, while the number of dead individuals remained minimal (2-8%) (Table 2). Furthermore, at doses of 100-1000 µg/mL, the effectiveness of CxEt manifested as an increase in the number of dead NEMs. After 24 and 72 h, the IC_{50} of CxEt toward NEMs was 13.4 and 0.62 µg/mL, respectively.

Effects of CxEt on Adult O. felineus

Adult flukes (maritae) kept in RPMI 1640 medium (negative control) without the addition of CxEt throughout the entire observation period (72 h) remained alive and active, and their motility declined only slightly by the end of the experiment (by 13%) (Table 1, Fig. 1A), with all individuals remaining alive (Table 2). The lowest CxEt dose (0.1 µg/mL) did not affect maritae motility within 3 days, but motility at all other concentrations was reduced compared to control values at each time point (Mann-Whitney U test).

At 3 h after the addition of CxEt, no changes in motility or mortality of maritae (n = 114) were detectable. Low concentrations of CxEt (0.1-10 µg/mL) throughout the entire observation period did not cause maritae death.

Maritae motility started to decrease 24 h after the addition of CxEt at doses of 400 µg/mL or higher (Table 1). On days 2 and 3, all maritae were immotile at high CxEt concentrations (400-1000 µg/mL) (Tables 1 and 2). After 24 h of exposure to either 100 or 200 µg/mL CxEt, similar effectiveness values in terms of motility were observed (RM = 49 and 53, respectively), with identical (8%) mortality rates of maritae (Table 2). At CxEt concentrations of 400 and 600 µg/mL, half of maritae died, and motility parameters were similarly reduced. The death of all maritae was observed at 1000 µg/mL. Within this observation period (24 h), intravital external destruction of the marita integument under the influence of CxEt was not detectable. The extract inhibited motility and caused clouding of the marita parenchyma and distortion of their bodies, all of which resulted in death (Fig. 1E-F). After 48 and 72 h, the effectiveness of CxEt was higher at 200 µg/mL than at 100 µg/mL in terms of both motility inhibition and mortality. After 48 h of exposure to either 400 or 600 µg/mL CxEt, maritae were completely immobilized, and after 72 h they were dead. CxEt concentrations of 800 and 1000 µg/mL were lethal to maritae within these observation periods (Table 2). After 24 and 72 h, the IC_{50} of CxEt toward maritae was 10.9 and 0.97 µg/mL, respectively.

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Table 1: Changes in the motility of O. felineus NEMs and maritae exposed to different CxEt doses on the 4-point scale

Doses of CxEt (μg/mL)	24 h mean ± S.E.M	48 h mean ± S.E.M	72 h mean ± S.E.M	96 h mean ± S.E.M	χ2 (df=9)	p-value
NEWLY EXCYSTED METACERCARIAE						
0 (n=191)	3.9±0.02	3.8±0.03	3.7±0.04	3.4±0.05		
0.1 (n=119)	3.9±0.03	3.0±0.09	2.0±0.10	2.0±0.09		
1 (n=123)	3.5±0.05	2.9±0.09	2.0±0.08	1.5±0.08		
10 (n=95)	3.9±0.04	3.1±0.10	2.1±0.10	1.9±0.07	1295.62 [a]	<0.001
100 (n=145)	3.3±0.07	2.5±0.06	1.1±0.03	1.1±0.02		
200 (n=175)	2.2±0.03	1.5±0.04	1.2±0.03	1.0±0	1125.24 [b]	<0.001
400 (n=175)	1.1±0.03	1.0±0	1.0±0	1.0±0	933.16 [c]	<0.001
600 (n=159)	1.1±0.02	1.0±0	1.0±0	1.0±0	1028.12 [d]	<0.001
800 (n=220)	1.0±0	1.0±0	1.0±0	1.0±0		
1000 (n=83)	1.0±0	1.0±0	1.0±0	1.0±0		
ADULT FLUKES						
0 (n=16)	3.8±0.09	3.6±0.10	3.3±0.09			
0.1 (n=10)	3.4±0.22	3.3±0.29	2.9±0.23			
1 (n=10)	3.0±0.28	2.8±0.23	2.5±0.15			
10 (n=12)	3.3±0.29	3.2±0.19	2.5±0.22			
100 (n=12)	2.6±0.34	2.04±0.23	1.8±0.21		77.32 [a]	<0.001
200 (n=10)	2.7±0.36	2.2±0.13	1.3±0.15		75.71 [b]	<0.001
400 (n=10)	1.1±0.10	1.0±0	1.0±0		72.19 [c]	<0.001
600 (n=10)	1.2±0.13	1.0±0	1.0±0			
800 (n=10)	1.2±0.13	1.0±0	1.0±0			
1000 (n=14)	1.0±0	1.0±0	1.0±0			

Motility was scored by the conventional method for *in vitro* experiments on helminths [22], described in Experimental design. Differences between groups after CxEt application were noted after ([a]) 24 h), ([b]) 48 h, ([c]) 72 h, ([d]) 96 h

Table 2: The time course of effects of various doses of CxEt on relative motility (percentage) and mortality (percentage) in NEMs and adult flukes of O. felineus

Doses of CxEt (μg/mL)	Relative motility (RM) (%) / Mortality (%)						
	24 h	48 h	72 h	96 h	24 h	48 h	72 h
	NEWLY EXCYSTED METACERCARIAE				*ADULT FLUKES*		
0	98/0	95/0	89/2	82/2	90/0	88/0	85/0
0.1	97/0	80/0	44/0	42/1	89/0	88/0	55/0
1	96/0	76/1	43/2	41/4	83/0	77/0	50/0
10	86/0	68/4	39/8	28/8	66/0	52/0	37/0
100	76/0	54/9	4/35	2/51	49/8	40/8	25/17
200	41/2	20/15	3/52	0/93	53/8	25/33	10/50
400	5/86	0/92	0/100	0/100	10/50	0/67	0/100
600	2/91	0/100	0/100	0/100	7/50	0/83	0/100
800	0/100	0/100	0/100	0/100	4/83	0/100	0/100
1000	0/100	0/100	0/100	0/100	0/100	0/100	0/100

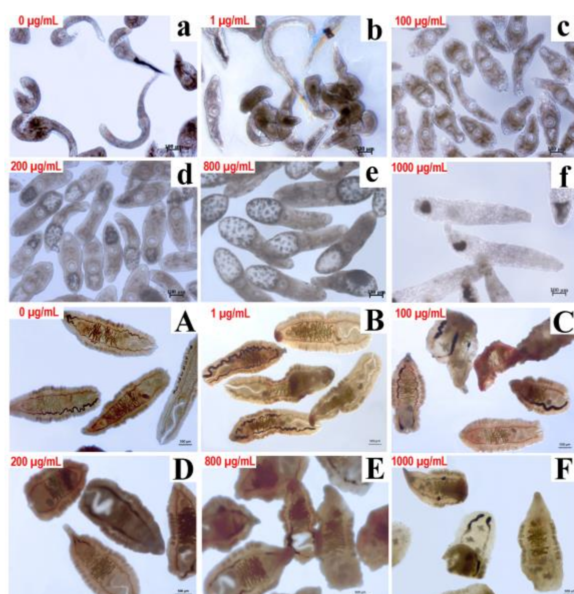
Levels of Secondary Metabolites in C. xanthiifolia

The leaves and seeds of the plant were found to contain elevated levels of triterpene saponins and total phenolic compounds (according to the Folin-Ciocalteu method), including tannins and phenolic acids (hydroxycinnamic acids), and moderate levels of flavonols and catechins (Table 3). In the DPPH assay, CxEt samples exhibited effective antiradical activity: at a concentration of 580 μg/mL, 50% of free radicals were eliminated (IC_{50} for antiradical activity of CxEt: 580 μg/mL).

Table 3: Quantitative phytochemical analyses of C. xanthiifolia

Secondary metabolite	Amount
Total tannins (mg TE/g)	32.05
Phenolic acids (mg CAE/g)	11.38
Total Phenolic Compounds (mg GAE/g)	8.20
Total Saponin (%)	4.03
Total Flavonol (mg RE/g)	1.73
Catechins (mg CE/g)	0.67

TE, Tannin equivalent; CAE, caffeic acid equivalent; GAE, gallic acid equivalent; RE, rutin equivalent; CE, catechin equivalent



(a) Control NEMs. Elongated bodies point to active movement.
(b) Some individuals are less elongated and darker.
(c, d) NEMs are shortened and more voluminous; the body does not elongate; the excretory bladder starts to enlarge.
(e) All NEMs are dead; the body is greatly enlarged; parenchyma is dark; tegument intact; the excretory bladder constitutes one-third of the body.
(f) All NEMs are dead; the look of the tegument and parenchyma points to degradation.
(A) Control adults have a symmetrical body and transparent parenchyma.
(B, C) The body is curved; spots of cloudy parenchyma appear.
(D) One-third of maritae are dead; they have an enlarged excretory bladder; the parenchyma is dark.
(E, F) The cloudy parenchyma and deformed body are signs of death.

Fig. 1: Morphology of (a-f) NEMs and (A-F) adult flukes after 24 h of exposure to various doses of CxEt

Evaluation of the Cytotoxicity of CxEt

The cytotoxic effect of CxEt on HFF10 cells was dose dependent (Figure 2). The calculated concentration of CxEt at which 50% of HFF10 cells died after 72 h (IC_{50}) was 168 μg/mL, and 95% of the cells died at 687 μg/mL, whereas the minimal toxic dose at which no more than 5% of the cells died was found to be 41 μg/mL (R^2 = 0.99).

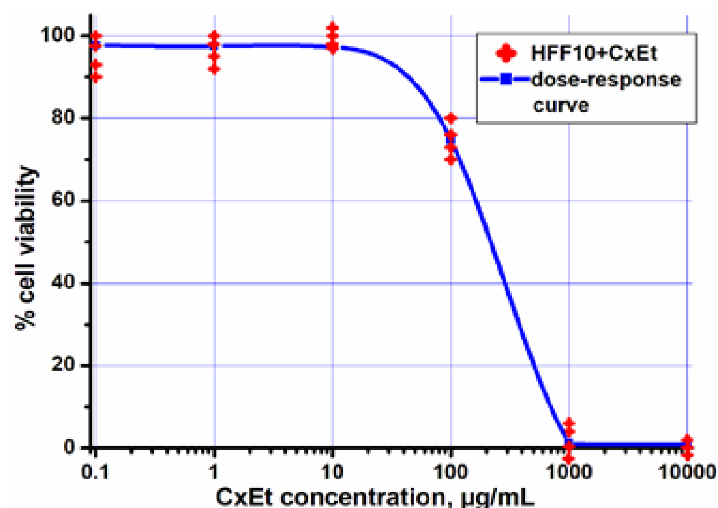


Fig. 2: Viability of HFF10 cells after 72-h incubation with CxEt, as determined by the MTT assay

Discussion

In our study, anthelmintic effectiveness of CxEt was evaluated by two parameters: relative motility (RM) and mortality of trematodes *O. felineus* at two stages of development (NEMs and maritae). This approach allowed us to identify dose- and time-dependent features of CxEt's action on the trematodes. For instance, CxEt began to inhibit NEMs motility (without causing mortality) within 3 h (especially at high concentrations, 800-1000 µg/mL), but in maritae, this effect was not detectable. After 24 h, high doses caused 100% mortality of NEMs. In comparison, complete immobility and mortality of maritae at these doses was observed later (48-72 h). Consequently, at high doses of CxEt, NEMs are more sensitive to this substance than *O. felineus* maritae. On the contrary, the reduction in relative motility of maritae exposed to low concentrations of CxEt (≤100 µg/mL) for 24 h was greater than that in NEMs.

The IC_{50} values determined for CxEt (NEMs: 13.4 µg/mL, adult worms: 10.9 µg/mL) are weaker than those previously reported for the clinically used praziquantel (IC_{50} 0.56 µg/mL for NEMs, 0.25 µg/mL for adult worms) [20]. Praziquantel is a chemically pure drug, whereas CxEt contains many phytochemicals extracted from *C. xanthiifolia*, and therefore a direct unconditional comparison of their effectiveness is not fully appropriate. Nonetheless, a comparative assessment of anthelmintic potential of crude plant extracts is the first stage of research prior to identification of the key active ingredient(s), animal experiments, and other downstream analyses.

A comparison of our results with data from other researchers is difficult at present. There are few findings reported in the literature regarding the effects of plant extracts on *O. felineus*. For instance, earlier, scientists from Siberia experimentally demonstrated the effectiveness of aspen bark extracts [29]. Later, extracts of four plant species from the genus *Centaurea* (cornflower) were tested. Antiopisthorchiasis activity was detected in a guaiane-type sesquiterpene lactone called cynaropicrin isolated from *Centaurea scabiosa* [10]. Antiopisthorchiasis properties of *Achillea millefolium* and *Achillea nobilis* were also confirmed experimentally [10]. Curcumin, isolated from *Curcuma longa* L., proved to be effective in vitro against NEMs and maritae of *O. felineus*, and its efficacy was comparable with that of praziquantel [30]. In addition, a potent schistosomicidal effect of curcumin has been demonstrated in several studies both in vitro and in vivo [31].

No studies on the effects of *C. xanthiifolia* extracts on other trematode species in vitro have been reported. On the other hand, J.F. Ferreira et al. [32] showed that a crude alcoholic extract of *Artemisia annua* (which has well-known antiparasitic properties) killed maritae of three trematode species (*Fasciola hepatica*, *Schistosoma mansoni*, and *Echinostoma caproni*) at a concentration of 2000 µg/mL within 24 h. According to our findings, after 1-day exposure to CxEt, *O. felineus* maritae are killed at approximately half that concentration (1000 µg/mL). A similar result was obtained in a preliminary screening of crude extracts of five plant species against the trematode *Gastrothylax crumenifer* [33]. Those authors selected a crude leaf extract of *Terminalia catappa* for further investigation because this extract at 1000 µg/mL caused 100% mortality of the parasite in vitro after 24 h. Accordingly, we believe that our initial data on the effects of CxEt are encouraging and promising for further investigation.

Given that a prospective anthelmintic agent, aside from being highly effective, should also be safe for the parasite's host, we assessed CxEt cytotoxicity. The death of 50% of normal human HFF10 fibroblasts occurred at a CxEt concentration of 168 µg/mL after 72 h, while the motility of 50% of NEMs ceased already at 0.62 µg/mL, and that of maritae at 0.97 µg/mL. As shown in Figure 2, all HFF10 cells within the concentration range of 0.1-41.0 µg/mL retained 95-100% viability after 72 h. Thus, the anthelmintic action of CxEt against *O. felineus* occurs at substantially lower concentrations than those causing irreversible effects in cultured mammalian cells. Our results indicate that the use of CxEt in further studies on laboratory animals is justified.

One of the advantages of plant-based anthelmintics is their multifunctionality, determined by the unique contribution of each component within a formulation. High concentrations of saponins and phenolic compounds, including tannins and phenolic acids (hydroxycinnamic acids), were detected in CxEt. It is likely that the anthelmintic effect of the extract is primarily associated with these constituents. Nevertheless, we cannot rule out that specific combinations of these compounds within CxEt may exert a synergistically enhanced anthelmintic effect compared to individual compounds; this topic should be addressed in future studies. Mechanisms of action of the constituents may differ and target different physiological systems (and may require different routes of administration) in the helminth body. In this regard, the combined action of extract components may offer an advantage over single-compound approaches. Currently, an increasing number of studies emphasize the potential of combination anthelmintic strategies involving compounds with different mechanisms of action in both experimental and clinical settings [9,22,34,35].

Tannins, which were present in high amounts in CxEt, are known to bind proteins, thereby disrupting physiological processes such as feeding, reproduction, and integument integrity in nematodes [36]. Condensed tannins interact with proline-rich proteins on the nematode integument, thus interfering with motility, feeding, and other key metabolic processes [37]. Saponins are plant-derived glycosides that interact with cell membranes by selectively complexing with cholesterol; they include glycosylated triterpenoids, steroids, and steroidal alkaloids. Many saponins exhibit antimicrobial effects, inhibit fungal growth, and protect plants from insects [38]. They have been reported to possess antioxidant properties and to be lethal to protozoa and nematodes [39,40]. In addition, saponins cause damage to the integument of the trematode *Cotylophoron cotylophorum* [41]. Hydroxycinnamic acids, aside from their well-known antioxidant activities, possess antitumor, antimalarial, antimicrobial, anti-inflammatory, hepatoprotective, and antidiabetic properties [42]. Some studies have also reported anthelmintic (ovicidal) activity of hydroxycinnamic acids isolated from *Chamaecrista nictitans* [43]. Consequently, secondary metabolites may not only exert toxic effects on helminths but also provide protective and antioxidant effects on host tissues [44].

It is possible that the combination of tannins, saponins, and hydroxycinnamic acids within CxEt contributes to its antiradical effect in the host organism. It is known that overproduction of reactive oxygen species during parasitic infections initially leads to activation of neutrophils, macrophages, and eosinophils, intended to eliminate protozoa or damage multicellular parasites at sites of inflammation [44]. At the cellular level, free radicals generated during oxidative stress can damage macromolecules by inducing lipid peroxidation, DNA fragmentation, and protein oxidation [44]. Therefore, the combination of CxEt's anthelmintic effect with its high antiradical activity represents a potentially beneficial dual function of the extract. It has been hypothesized that there is a direct correlation between antioxidant potential and anthelmintic activity of plant extracts [45], as demonstrated in studies on the anthelmintic properties of an extract from *Glinus oppositifolius* [46].

Taking into account our results on the opisthorchicidal action of CxEt, we suggest that further research on the anthelmintic activity of secondary metabolites of CxEt in vitro is necessary, as well as studies on their effects in laboratory animals as models of opisthorchiasis. However, reproducibility of experiments across different laboratories may be affected by variation in *C. xanthiifolia* growth regions and collection seasons. These factors may influence the efficacy of anthelmintic activity.

Conclusion

C. xanthiifolia can be considered a candidate natural anthelmintic agent against metacercariae and maritae of the trematode *O. felineus*. Due to the high concentrations of tannins, saponins, and hydroxycinnamic acids in the extract from this plant, as well as its effective antiradical activity and low cytotoxicity, the extract of *C. xanthiifolia* can be used in subsequent in vivo studies. With regard to the anthelmintic action of CxEt on NEMs and the absence of cytotoxicity, we do not preclude the use of the extract in preventing infection with *O. felineus*, an approach not feasible with the clinically used medication praziquantel. This will be the subject of our further investigation.

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Author's Contributions

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Elena Khramova: Methodology, Editing, and Validation. Natalia Gubanova: Data curation, Methodology, Editing, and Validation.
Tatyana Shaldaeva: Data curation, Resources, and Methodology.
Irina Chidunchi: Methodology and Software.
Damira Avgustinovich: Conceptualization, Project administration, Editing, and Validation.

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