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Comparative Study of *Plantago media* Extracts in the Treatment of *Acanthamoeba* sp. Trophozoites

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Abstract: (1) Background: The aim of the study was to compare the potency of *Plantago media* L. (Plantaginaceae) extracts on *Acanthamoeba* sp. trophozoites, which are opportunistic protozoan parasites leading to several dangerous diseases; (2) Methods: The chromatographically (TLC, HPLC-DAD) characterized water fractions of the extracts from biomass from in vitro cultures (shoots and roots), leaves, and inflorescences from field cultivation were used for the study of the acanthamoebic activity in a Thoma haemocytometer chamber; (3) Results: The anti-amoebic effect at the lowest concentration (1.0 mg/mL) was demonstrated only by the extract of the leaves from the cultivation (50.50% inhibition). The remaining samples inhibited the growth of parasites from a concentration of 5.0 mg/mL in the range of 41.36% inflorescences to 63.89% shoots in vitro. Quantitative determinations of phenolic compounds in the tested extracts indicate a tendency to increase the potency of the anti-amoebic effect with the content of a phenylethanoid glycoside—acteoside. The maximum content of this compound was determined in leaves from field cultivation (6.64%) and the minimum in inflorescences (0.65%). This is confirmed by the range of the lowest IC_{50} values (the strongest biological activity) for the tested samples, 0.95–1.80 mg/mL for leaves from cultivation, and the high values, 9.70–5.30 mg/mL for inflorescences and in-vitro-derived roots. The strength of the biological activity of the extracts correlated with the content of acteoside, which constituted 84–93% of the sum of phenolic compounds determined; (4) Conclusions: The performed investigations proved the anti-acanthamoebic efficacy of *Plantago media* organs, including those obtainable by biotechnological methods, and indicated phenylethanoid glycosides, their main phenolic constituents, to be responsible for the activity. To our knowledge, this is the first report on the amoebicidal activity of *Plantago media* extracts from biomass produced by biotechnological methods and organs of an intact plant.

Keywords: *Plantago media*; in vitro cultures; plant phenolics; phenylethanoid glycosides; acteoside (verbascoside); acanthamoebic activity

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1. Introduction

Plantago media L., the horary plantain, belongs to the Plantaginaceae family, and is a representative of the genus *Plantago* L., which contains 242 species of herbaceous plants, or, less often, semi-shrubs, characterized by a cosmopolitan occurrence [1,2]. *P. media* (the leaves were the commonly used part) had several applications in folk medicine in the past and, now, many of its medical activities have been scientifically proven, such as its antioxidant, antitumoral, cytotoxic, anti-allergenic, mycostatic, anti-inflammatory, and tyrosine kinase inhibitor properties [3]. Several other *Plantago* species are known medicinal plants, such as *P. major*, *P. asiatica*, *P. lanceolata*, and *P. ovata* [4], and some of them have

monographs in pharmacopoeias, e.g., *P. lanceolata*, and *P. ovata* [5]. Plants of the genus *Plantago* can be used in medicine due to their anti-inflammatory, analgesic, anti-cancer, antiviral, and anti-ulcer effects; they also have expectorant and diuretic properties [4,6–9].

The main groups of compounds with a wide spectrum of biological activity that can be found in *P. media* are phenylethanoid glycosides [10–12], iridoid glucosides [13,14], phenolic acids [15,16], flavonoids [15,17], and polysaccharides [12,18]. In the micropropagated plants of *P. media,* there were mainly phenylethanoid glycosides present, and no flavonoids were detected [19].

The phenylethanoid glycosides occurring in *Plantago* are represented mainly by acteoside (verbascoside) and also its positional isomer—isoacteoside, which is a water-soluble, low-molecular secondary metabolite [10,11,20]. The phenylethanoid glycosides are associated with a wide range of biological and pharmacological activities [21–23]. Acteoside and isoacteoside are known for their prominent antiviral activity and, as suggested by recent literature reports, are likely to possess an inhibitory effect against SARS-CoV-2 [24]. Acteoside has been recognized as an effective scavenger of free radicals and an inhibitor of lipid peroxidation [25]. It exhibits neuroprotective activity through cholinergic, anti-inflammatory, and antioxidant mechanisms [26]. In addition, it shows an antiproliferative effect in many cancer cells [27]. What is particularly interesting is that the pronounced antiprotozoal activity against *Leishmania donovani* and trypanocidal activity against *Trypanosoma brucei* rhodesiense have been reported for acteoside and its isomer—isoacteoside [21]. More recently, acteoside was shown to inhibit *Leishmania amazonensis* by the inhibition of an enzyme arginase of extracellular promastigotes [28] and intracellular amastigotes [29].

The opportunistic pathogens of *Acanthamoeba* species have become recognized as dangerous agents leading to serious diseases, including granulomatous amoebic encephalitis (GAE), amoebic keratitis (AK), and amoebic pneumitis (AP). The single-cell free-living organisms commonly occur in the environment and are found in soil, air, and fresh and salt water. It is easy to become infected because their place of existence can also be air-conditioning systems, showers, sanitary appliances, swimming pools, and dialysis units. They were discovered in bottled mineral water and contact lens fluids. *Acanthamoeba* trophozoites are metabolically and productively active in the appropriate conditions; however, they have the ability to transform into dormant cysts in a stressful environment [30]. The treatment of amoebic diseases is usually long-lasting and not very effective. Plant-derived natural products remain a promising source for drug development [30–35].

The report on the use of an acteoside-containing traditional medicinal plant species *Stachytarpheta cayanensis* (Rich.) Vahl from the family Verbenaceae (such as Plantaginaceae, classified within the order Lamiales) against a protozoan parasite *Leishmania* [28] has prompted us to investigate *Plantago media* for anti-acanthamoebic activity.

The aim of the present study was to evaluate the phenolic compound (phenylethanoid glycosides, phenolic acids, and flavonoids) content and to investigate the in vitro anti-amoebic activity of the *Plantago media* extracts obtained from biomass produced by biotechnological methods, i.e., plant in vitro cultures (shoots and roots), as well as organs from an intact plant, on the growth and development of *Acanthamoeba* sp. trophozoites. The extract of *P. lanceolata* leaves—a pharmacopoeial raw material [5,36]—was used for comparisons.

2. Materials and Methods

2.1. Plant Material

The voucher specimen of *Plantago media* L. (no. PM001/2012) and of *Plantago lanceolata* L. (no. PLK20060615) is deposited in the Herbarium of Laboratory of Pharmaceutical Biology and Plant Biotechnology (previously Department of Pharmaceutical Botany and Plant Biotechnology), Poznan University of Medical Sciences, Poland. The biomass from in vitro conditions were shoots and roots obtained from micropropagated plantlets multiplied via shoot tip cultures on Murashige and Skoog medium with 6-benzylaminpurine 2.0 mg/mL according to the protocol evaluated previously [19].

Leaves and inflorescences of *P. media*, as well as leaves of *P. lanceolata*, were collected in May and June from the botanical garden of the Department of Medicinal and Cosmetic Products, Poznan University of Medical Sciences (52°25′30.7″ N, 16°53′50.8″ E).

2.2. Preparation of Extracts

The preparation of extracts from the plant materials for the purposes of profiling phenolic compounds' presence by TLC analysis and anti-acanthamoebic activity evaluation was described in Section 2.3. The extracts for quantitative analysis by HPLC were obtained as described in Section 2.4. Methanol was chosen as extracting agent. Methanol is the most universal solvent for plant material extraction, because of its wide power of metabolite dissolution, both hydrophilic and lipophilic; very low risk of decomposition of extracted compounds (absence of water); and easy evaporation. Ethanol is also a very good solvent, but less polar and requires the addition of water to ensure extraction of more polar compounds, such as flavonoid glycosides. Boiling water extraction provides unstable water extracts, which require immediate processing. A similar situation, albeit even more complicated, occurs with expressed juices due to the possibility of the decomposition of compounds by endogenous enzymes.

2.3. TLC Analysis

Dried plant material was extracted for 1 h three or four times with boiling methanol (flowers and biomass from in vitro cultures) or with 80–20% aqueous acetone (leaves of *P. media* and *P. lanceolata*). The solid/liquid ratio was 1:15. The combined extracts were evaporated at 40 °C to dryness using rotary vacuum evaporator, weighted, and then partitioned into dichloromethane and water fractions. Solutions of water fractions in 70% ethanol (0.1 g/mL) were analysed by one- or two-dimensional thin-layer chromatography (1D- and 2D-TLC) [37]. Phenolic compounds (phenylethanoid glycosides)—acteoside, plantamajoside, martynoside, and lavandulifolioside isolated from *Plantago lanceolata* [38], as solutions in ethanol (1.0 mg/mL)—were used as reference samples for comparison. The fractions and standard solutions were applied to TLC plates in 5 µL volumes. The used stationary and mobile phases were: silica gel (pre-coated, Merck, Darmstadt, Germany) developed with *n*-butanol-acetic acid-water 4:1:5 (*v*/*v*/*v*, organic layer) (BAW) or ethyl acetate-acetic acid-water 4:1:1 (*v*/*v*/*v*); cellulose (pre-coated, Merck) developed two-dimensionally with BAW followed by 15% acetic acid (AcOH); and polyamide DC6 (home-made, Macherey-Nagel, Düren, Germany) developed with ethyl acetate-ethanol-water 20:3:2 (*v*/*v*/*v*) or water-*n*-butanol-acetone 16:3:3 (*v*/*v*/*v*). The compounds on chromatograms were visualized under daylight and/or UV 366 nm and 254 nm light before and after spraying with a reagent for general detection of phenolics: 0.1% NA (2-aminoethanol diphenylborate, Roth, Karlsruhe, Germany) in ethanol or a reagent for detection of flavonoids, and 1% aluminum chloride in ethanol followed by heating [39,40]. The phenylethanoid glycosides were detected by UV as blue spots showing strong blue fluorescence after NA spraying. The flavonoids were recognized under UV light as brown spots changing color to brilliant yellow fluorescence with aluminum chloride as well as yellow or orange fluorescence with NA [41,42].

2.4. HPLC-DAD Analysis

The leaves and inflorescences from intact plants and shoot and root biomasses from in vitro cultures were dried at 40 °C for 24 h to a constant weight. Each 0.5 g dry weight (DW) sample of the dried and pulverized material was subjected to sonication (Polsonic® 3, Warsaw, Poland) extraction with methanol (5 mL) for 30 min three times. For the analyses of flavonoids and phenolic acids, the validated HPLC-DAD method, described by us earlier, was employed [43,44]. The identification of compounds was based on Rt (retention time) values, UV-DAD spectra (λ = 200–400 nm), and the method of internal reference standard addition. Quantification was performed by a comparison of peak area measurements to those of the standard curves. The following standards were used: vanillic acid, protocatechuic acid, gallic acid, caffeic acid, syringic acid, ferulic acid, isoferulic acid, acteoside,

isoacteoside, quercetin, apigenin, isorhamnetin, rutoside, and luteolin were purchased in Sigma-Aldrich (Saint Louis, MO, USA) company.

2.5. Anti-Amoebic Activity

This study used the *Acanthamoeba* sp. strain Ac55 (isolated from a patient with keratitis, T4 genotype) deposited in GenBank (NCBI) under accession number KP120880. The axenic cultures of amoebae were performed on a liquid medium containing 2% Bacto-Casitone. The dried water fractions of the extracts were dissolved in 50 µL of dimethyl-sulfoxide (DMSO) and further diluted with distilled water to achieve the appropriate concentrations. The final concentration of DMSO was 0.0001–0.0002 µL/mL of liquid amoeba culture. These dilutions were added to the amoebae culture with density of 5×10^4 cells/mL at concentrations of 1–10 mg/mL. The changes in the number of amoebae were checked at 24 h intervals for three days in a Thoma haemocytometer chamber (Hirschmann, Eberstadt, Germany). The control consisted of cultured trophozoites without fractions. The relationship between fraction concentration and changes in the growth of trophozoites during the time of treatment of trophozoite cultures was investigated.

2.6. Statistical Analysis

Data are expressed as means and standard deviation (±SD). The number of repeated experiments was $n = 18$ for biological determinations, and $n = 3$ for HPLC determinations. The IC_{50} values were read from the plots of *Acanthamoeba* growth inhibition against concentration of the tested extract. The collected data were subjected to a one-way analysis of variance (ANOVA) followed by Duncan's post-hoc test. A two-sided p-value of 0.05 was applied to declare statistical significance. Statistical analysis was performed by using the Statistica software program ver. 13 (Statsoft, Kraków, Poland).

3. Results

The protocol of efficient micropropagation of *Plantago media* L. was developed previously [19]. The shoots and roots of in vitro micropropagated plantlets were chosen for the experiments to compare whether raw materials produced by biotechnological methods are richer in selected secondary metabolites and have higher biological activity, in this case, acanthamoebic. Moreover, to compare the activity of extracts resulting from the presence and content of bioactive compounds, *Plantago media* was compared to the well-known and valued raw material of *Plantago lanceolatae folium* (Figure 1).

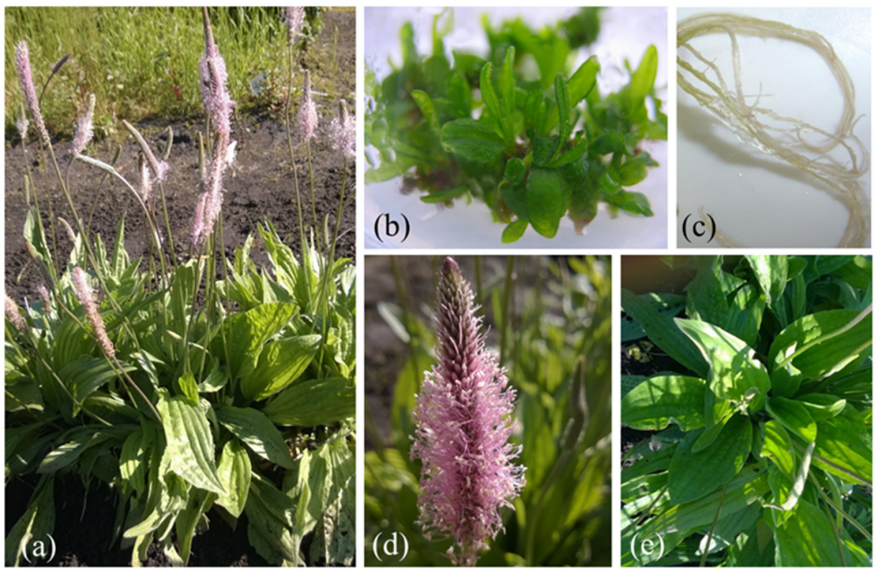


Figure 1. Plant materials used for the investigations: *Plantago media* in soil cultivation (**a**) with zoomed inflorescences (**d**) and leaves (**e**); and *P. media* in-vitro-derived leaves (**b**) and roots (**c**).

Chromatographic examination of extracts by 2D-TLC fingerprinting on cellulose plates (Table 1) exhibited a similar pattern of phenolic compounds in shoots and roots of the micropropagated plantlets. Phenylethanoid glycosides, such as acteoside and plantamajoside, were present, and no flavonoids could be detected. The phenylethanoids appeared under UV light as blue bands exhibiting strong blue fluorescence after spraying with NA. In turn, no yellow or brown bands changing color to brilliant yellow fluorescence under UV upon derivatization with aluminum chloride, characteristic for flavonoids, were observed. The flavonoids were apparent in the extracts of intact plants—*P. media* inflorescences and leaves and *P. lanceolata* leaves (Table 1).

Table 1. TLC-fingerprinting analysis of phenolic compounds in *Plantago media*.

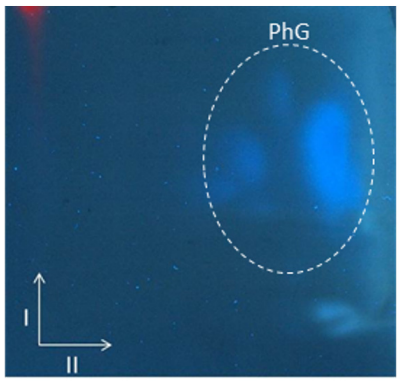
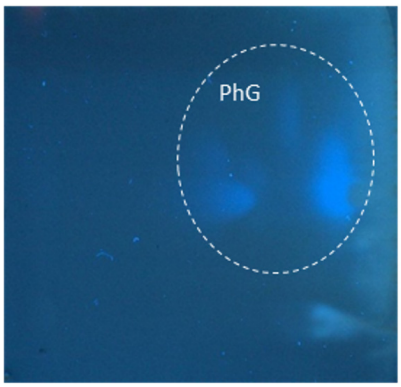
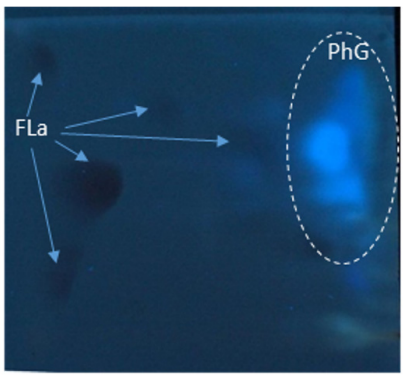
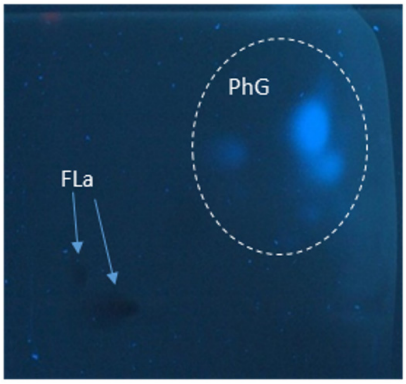
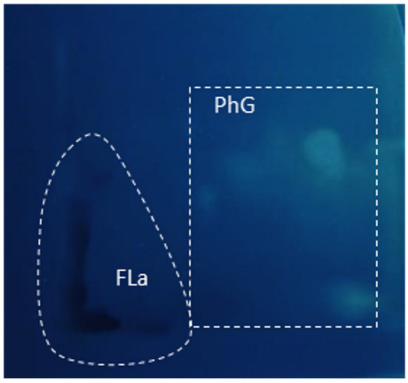
Material (Water Fraction of an Extract)	2D-TLC Cellulose (Adsorbent: Cellulose; Mobile Phases: I: BAW 4:1:5, II: 15%AcOH; Detection—UV 365 nm)	Flavonoids	Phenylethanoid Glycosides
Plantago media in vitro shoots		Absent	Present
Plantago media in vitro roots		Absent	Present
Plantago media inflorescences		Present	Present

Table 1. *Cont.*

Material (Water Fraction of an Extract)	2D-TLC Cellulose (Adsorbent: Cellulose; Mobile Phases: I: BAW 4:1:5, II: 15%AcOH; Detection—UV 365 nm)	Flavonoids	Phenylethanoid Glycosides
Plantago media leaves		Present	Present
Plantago lanceolata leaves		Present	Present

Fla—flavonoids (brown spots); and PhG—phenylethanoid glycosides (blue spots).

The results of quantitative analyses performed using the HPLC-DAD method indicate differences in the content of individual compounds—phenylethanoids, flavonoids and phenolic acids—depending on the type of *Plantago media* L. raw material (Table 2).

The leaves of plants from the ground and from in vitro cultures possess the highest content of the sum of phenylethanoids as well as individual compounds—acteoside and isoacteoside. Moreover, the content of acteoside is more than two-fold higher in the leaves of the plant from the ground (6641.70 ± 12.06 mg/100 g DW) than from the shoot culture (3034.11 ± 15.54 mg/100 g DW). Roots from in vitro cultures contain lower contents of acteoside (1039.31 ± 9.18 mg/100 g) and isoacteoside (161.93 ± 6.42 mg/100 g), although more than inflorescences (655.13 ± 5.16 mg/100 g DW and 67.68 ± 2.50 mg/100 g DW, respectively), which are the least rich in compounds, both phenylethanoids and phenolic acids. In general, it can be observed that acteoside is the dominant compound in each of the raw materials of this species, regardless of the type of organ (leaf, root, or inflorescence) or origin (organs of natural plants or biomass from biotechnological production). Acteoside and isoacteoside (phenylethanoids) are present in raw materials in a much higher concentration than the sum of phenolic acids or the sum of flavonoids. The content of acteoside constitutes 87.76% and 84.15% (in vitro shoots and roots), 84.96% and 93.83% (in *P. media* inflorescences and leaves), and 93.60% (in *P. lanceolata* leaves) of the phenolics sum content. For comparison, the content of these compounds in *Plantaginis lanceolatae folium*, a well-known pharmacopoeial raw material, was also examined and it turned out that the leaves of *P. media* are richer in acteoside and isoacteoside than the leaves of *P. lanceolata* (Table 2).

Table 2. The content of phenolic compounds [mg/100 g DW ± SD] in extracts of biomass from in vitro cultures (shoots and roots) and organs of plants from the field cultivation.

Compounds	*P. media* In Vitro Shoots	*P. media* In Vitro Roots	*P. media* Inflorescences	*P. media* Leaves	*P. lanceolata* Leaves
Phenolic acids					
Gallic acid	22.91 ± 0.08 ^{a}	17.14 ± 0.09 ^{b}	10.97 ± 0.12 ^{d}	11.40 ± 0.13 ^{c}	-
Protocatechuic acid	12.24 ± 0.13 ^{b}	1.08 ± 0.01 ^{d}	7.72 ± 0.15 ^{c}	24.76 ± 0.08 ^{a}	8.99 ± 1.26 ^{c}
Vanillic acid	15.86 ± 0.98 ^{a}	5.26 ± 0.21 ^{c}	4.53 ± 0.67 ^{d}	8.00 ± 0.50 ^{b}	-
Caffeic acid	5.96 ± 1.41 ^{a}	3.51 ± 0.98 ^{bc}	1.28 ± 0.04 ^{d}	2.49 ± 0.68 ^{c}	-
Syringic acid	7.94 ± 1.55 ^{a}	6.87 ± 0.99 ^{a}	2.12 ± 0.09 ^{b}	6.77 ± 0.34 ^{a}	-
Ferulic acid	-	-	-	-	7.28 ± 1.34
Isoferulic acid	-	-	-	-	34.86 ± 2.13
Sum	64.91	33.86	26.62	53.42	51.13
Phenylethanoids					
Acteoside	3034.11 ± 15.54 ^{c}	1039.31 ± 9.18 ^{d}	655.13 ± 5.16 ^{e}	6641.70 ± 12.06 ^{a}	4015.60 ± 40.2 ^{b}
Isoacteoside	358.15 ± 7.03 ^{a}	161.93 ± 6.42 ^{d}	67.68 ± 2.50 ^{e}	278.55 ± 4.64 ^{b}	185.06 ± 8.08 ^{c}
Sum	3392.26	1201.24	722.81	6920.25	4200.66
Flavonoids					
Quercetin	-	-	2.00 ± 0.12	11.95 ± 0.58	2.24 ± 0.68
Isorhamnetin	-	-	15.02 ± 0.65	82.33 ± 4.82	-
Apigenin	-	-	4.66 ± 0.29	10.57 ± 0.43	11.18 ± 0.39
Luteolin	-	-	-	-	1.23 ± 0.61
Rutoside	-	-	-	-	23.48 ± 0.61
Sum	0	0	21.68	104.85	38.16
Sum of all compounds	3457.17	1235.10	771.11	7078.52	4289.95

Mean values within a row with the same letter are not significantly different at $p < 0.05$ using Duncan's multiple range test—the first letter of the alphabet for the highest values, the next for statistically significant decreasing values.

The highest level of the sum of phenolic acids occurs, as with phenylethanoids, in the leaves, then the roots, and the least in the inflorescences of this species. In this case, the content of phenolic acids is higher in the leaves of the shoot culture (64.91 mg/100 g DW) than of the ground plant (53.42 mg/100 g DW). The predominant phenolic acid in the raw materials was protocatechuic acid (highest 24.76 ± 0.08 mg/100 g DW), gallic acid (highest 22.91 ± 0.08 mg/100 g DW), and vanillic acid (highest 15.86 ± 0.98 mg/100 g DW). The content of gallic acid and vanillic acid was two-fold higher in leaves from in vitro cultures than from a plant from the ground, while protocatechuic acid was two times more in leaves from shoot culture than in plants from the ground. The qualitative and quantitative composition in the range of phenolic acids of the leaves of *P. media* differed significantly from that in the leaves of *P. lanceolata*. In both raw materials, there was protocatechuic acid, but there was almost three times more in *P. media* than in *P. lanceolata*, in which ferulic and isoferulic acids were present (Table 2). In line with the 2D-TLC analysis, HPLC-DAD showed no flavonoids in the in-vitro-derived organs, in contrast to the soil-derived plants. These compounds were most abundant in *P. media* leaves (104.83 mg/100 g DW), followed by *P. lanceolata* leaves and *P. media* inflorescences (38.16 and 21.68 mg/100 g DW, respectively). *P. media* inflorescences and leaves contained the same compounds, the flavonols quercetin and isorhamnetin, and a flavone, apigenin, and the same principal constituent, isorhamnetin (15.02 ± 0.65 and 82.33 ± 4.82 mg/100 g DW, respectively). The *P. lanceolata* leaves shared quercetin and apigenin, but differed in the presence of the flavone luteolin and rutoside (quercetin diglycoside) as a major compound (23.48 ± 0.61 mg/100 g DW).

The results of the study indicated that the extracts obtained from *Plantago*, both from in vitro shoots and roots as well as from organs of intact plants, inhibited the growth of

Acanthamoeba sp. trophozoites to varying degrees. The dependence of the effect on the extract concentration and treatment time was noted (Tables 3 and 4) (Figures 2–4).

Table 3. Effect of extracts from *Plantago media* biomass from in vitro culture (shoots and roots) and organs of plants from the cultivation on inhibition of *Acanthamoeba* trophozoites during four-day treatment.

Plant Material	Extract Concentration	Duration of Treatment [Days]					
		2nd Day		3rd Day		4th Day	
		MN ± SD	% GI	MN ± SD	% GI	MN ± SD	% GI
Plantago media in vitro shoots	control	12.44 ± 2.22	0	22.94 ± 3.39	0	28.38 ± 4.33	0
	1 mg/mL	12.18 ± 2.77	2.10	18.41 ± 4.31	19.75	21.82 ± 6.80	23.11
	5 mg/mL	6.33 ± 1.98 *	49.06	11.42 ± 5.07 *	50.22	10.25 ± 3.47 *	63.89
	10 mg/mL	2.94 ± 1.61 *	76.27	4.20 ± 2.14 *	81.70	5.27 ± 5.66 *	81.44
Plantago media in vitro roots	control	12.44 ± 2.22	0	18.94 ± 3.39	0	27.38 ± 4.33	0
	1 mg/mL	9.18 ± 2.55	26.21	13.00 ± 2.94	31.37	16.67± 1.25 *	39.12
	5 mg/mL	8.83 ± 1.67	29.02	10.25 ± 3.49 *	45.89	13.91 ± 4.46 *	49.20
	10 mg/mL	6.03 ± 1.91 *	51.45	9.09 ± 2.35 *	52.01	12.00 ± 2.52 *	56.18
Plantago media inflorescences	control	12.12 ± 2.82	0	26.44 ± 6.04	0	28.00 ± 5.35	0
	1 mg/mL	10.76 ± 1.92	11.23	25.00 ± 4.97	5.45	26.27 ± 3.22	6.18
	5 mg/mL	9.83 ± 2.65	18.90	16.33 ± 3.73 *	38.24	16.42 ± 4.35 *	41.36
	10 mg/mL	4.67 ± 2.38 *	61.47	8.00 ± 2.47 *	69.75	8.10 ± 3.97 *	71.04
Plantago media leaves	control	12.12 ± 2.82	0	23.44 ± 6.04	0	32.00 ± 5.35	0
	1 mg/mL	6.00 ± 1.97 *	50.50	12.33 ± 3.89 *	47.40	17.67 ± 5.03 *	44.79
	5 mg/mL	5.91 ± 1.73 *	51.24	10.25 ± 3.92 *	56.28	14.50 ± 4.03 *	54.69
	10 mg/mL	5.05 ± 2.39 *	58.34	8.81 ± 3.43 *	62.42	12.40 ± 3.88 *	61.25
Plantago lanceolata leaves	control	10.11 ± 2.05	0	25.72 ± 4.36	0	38.39 ± 7.77	0
	1 mg/mL	9.76 ± 2.60	3.47	18.67 ± 4.88	27.42	14.00 ± 5.18 *	63.54
	5 mg/mL	8.67 ± 1.97	14.25	16.35 ± 4.83 *	26.57	10.25 ± 3.47 *	74.31
	10 mg/mL	1.33 ± 0.94 *	86.85	3.51 ± 2.32 *	86.35	5.27 ± 5.66 *	86.27

* $p < 0.05$, statistically significant difference in comparison with the control during the same time interval; $n = 18$. MN—mean number of trophozoites, and GI—growth inhibition.

Table 4. Determination of IC_{50} [mg/mL] for the studied extracts of *P. media* and *P. lanceolata*.

IC_{50}	*P. media* In-Vitro -Derived Shoots	*P. media* In-Vitro -Derived Roots	*P. media* Inflorescences	*P. media* Leaves	*P. lanceolata* Leaves
2nd day	5.20	9.70	8.70	0.95	7.65
3rd day	5.00	7.60	6.80	1.25	7.25
4th day	3.30	5.30	6.30	1.80	0.55

IC_{50}—the half-maximum anti-amoebic inhibitory concentration.

The extract of leaves from shoot cultures was ineffective on *Acanthamoeba* trophozoites at the lowest concentration (1 mg/mL) because no statistical differences were observed compared to the control, regardless of the duration of treatment. Higher concentrations (5 mg/mL and 10 mg/mL) of the extract lowered the average number of trophozoites and, for them, on the fourth day of treatment, the growth inhibition was 63.89% and 81.44%, respectively (Table 3).

For the in vitro root extract, there is a clear tendency for the activity to be dependent on the time of exposure. On the second day, only the extract at the highest concentration worked; on the third day, extracts at the lowest and medium concentrations; and on the fourth day, extracts in all three concentrations were effective. However, the highest trophozoites growth inhibition was around 50–60%.

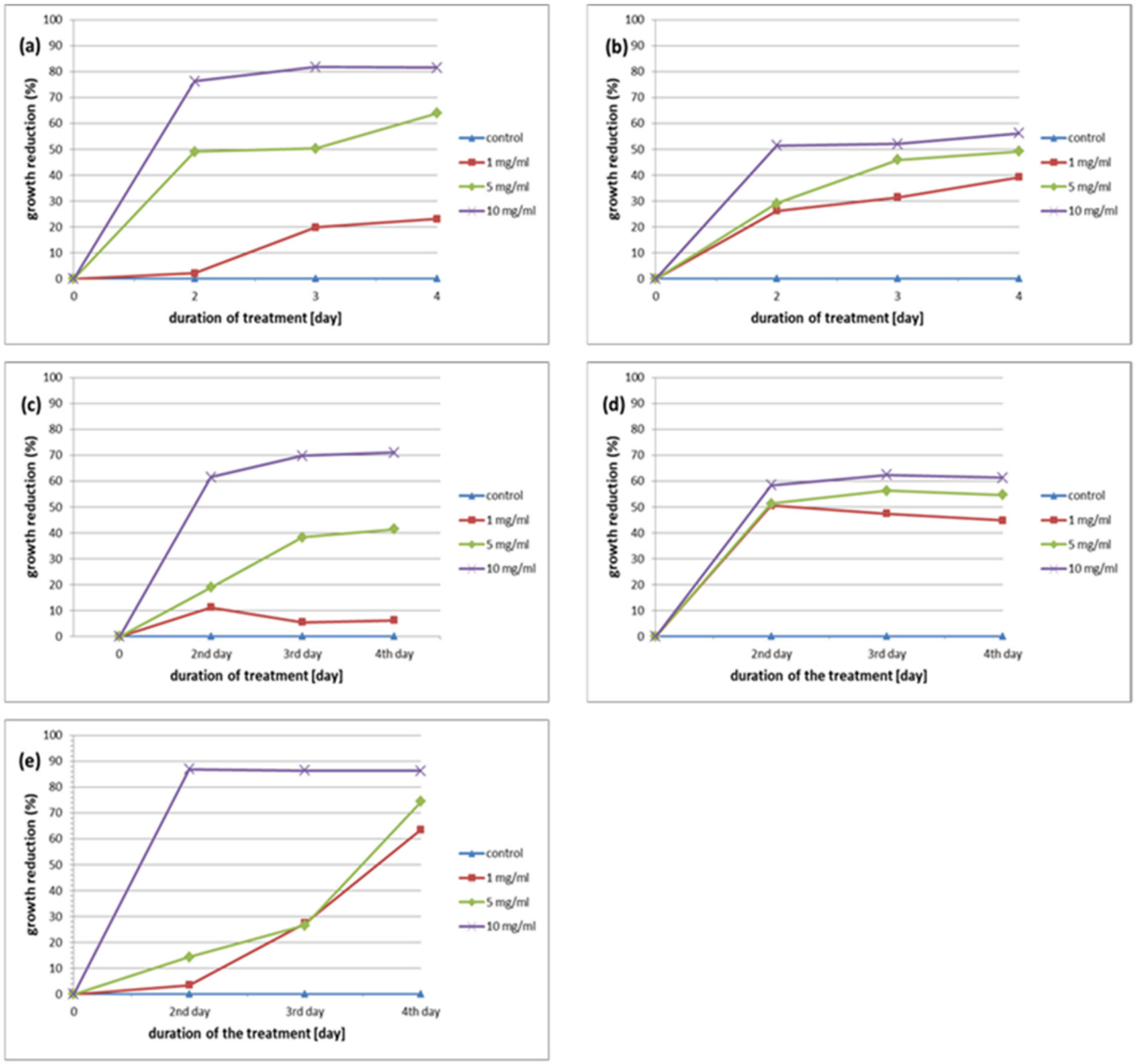


Figure 2. Effect of the extracts from *Plantago media* in vitro shoots (**a**), in vitro roots (**b**), inflorescences (**c**) and leaves (**d**), and *Plantago lanceolata* leaves (**e**) on the growth of *Acanthamoeba* sp.

In the case of the inflorescences extract, the dependence of the effect on the extract concentration and treatment time was noted. The highest percentage of growth inhibition of *Acanthamoeba* trophozoites was noticed for the extract at a concentration of 10 mg/mL (61.47% on the second day, 69.75% on the third day, and 71.04% on the fourth day). The leaf extract showed antitrophozoic activity at all concentrations.

Similar to the root extract of *P. media*, there is a clear tendency for the activity of the leaf extract of *P. lanceolata* to be dependent on the time of exposure. The highest *Acanthamoeba* trophozoites growth inhibition was noticed after incubation with the extract at 10 mg/mL (on the second day 86.85%, on the third day 86.35%, and on the fourth day 86.27%) (Table 3).

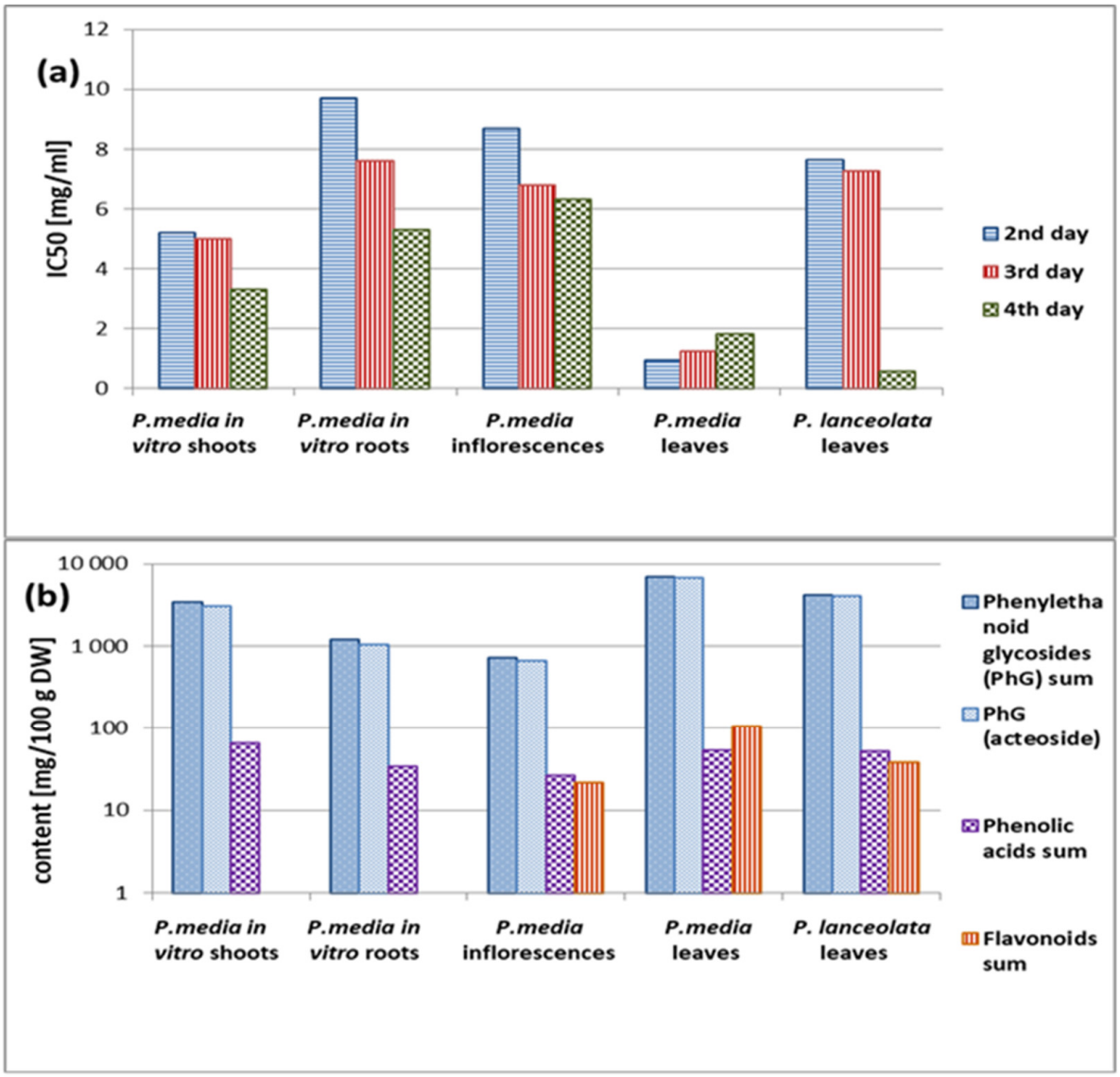


Figure 3. Comparison of IC_{50} values of anti-acanthamoebic activity (**a**) with the content of phenolic secondary metabolites in the investigated organs of *Plantago* (**b**).

The graphical comparison shown in Figure 3 suggested that IC_{50} values and the contents of phenolic constituents in the *Plantago* organs are inversely correlated. To check the nature of this correlation, the data were subjected to a Pearson test (Figure 4).

The analysis of the Pearson correlation coefficients (Figure 4) revealed the strongest correlation between IC_{50} and the content of the phenylethanoid glycosides sum and acteoside, followed by that of flavonoids sum, and the weakest correlation was found for the phenolic acids sum (mean r values were -0.8539 ± -0.84, -0.8506 ± -0.84, -0.7113 ± -0.68, and -0.6016 ± -0.58, respectively). The correlation with all phenolics sum was r -0.8541 ± -0.84, i.e., equal to that of phenylethanoid glycosides and very close to that of acteoside. The negative sign of the Pearson coefficients (Figure 4) proves that IC_{50} values and phenolics contents are inversely correlated—the higher the content, the stronger the activity.

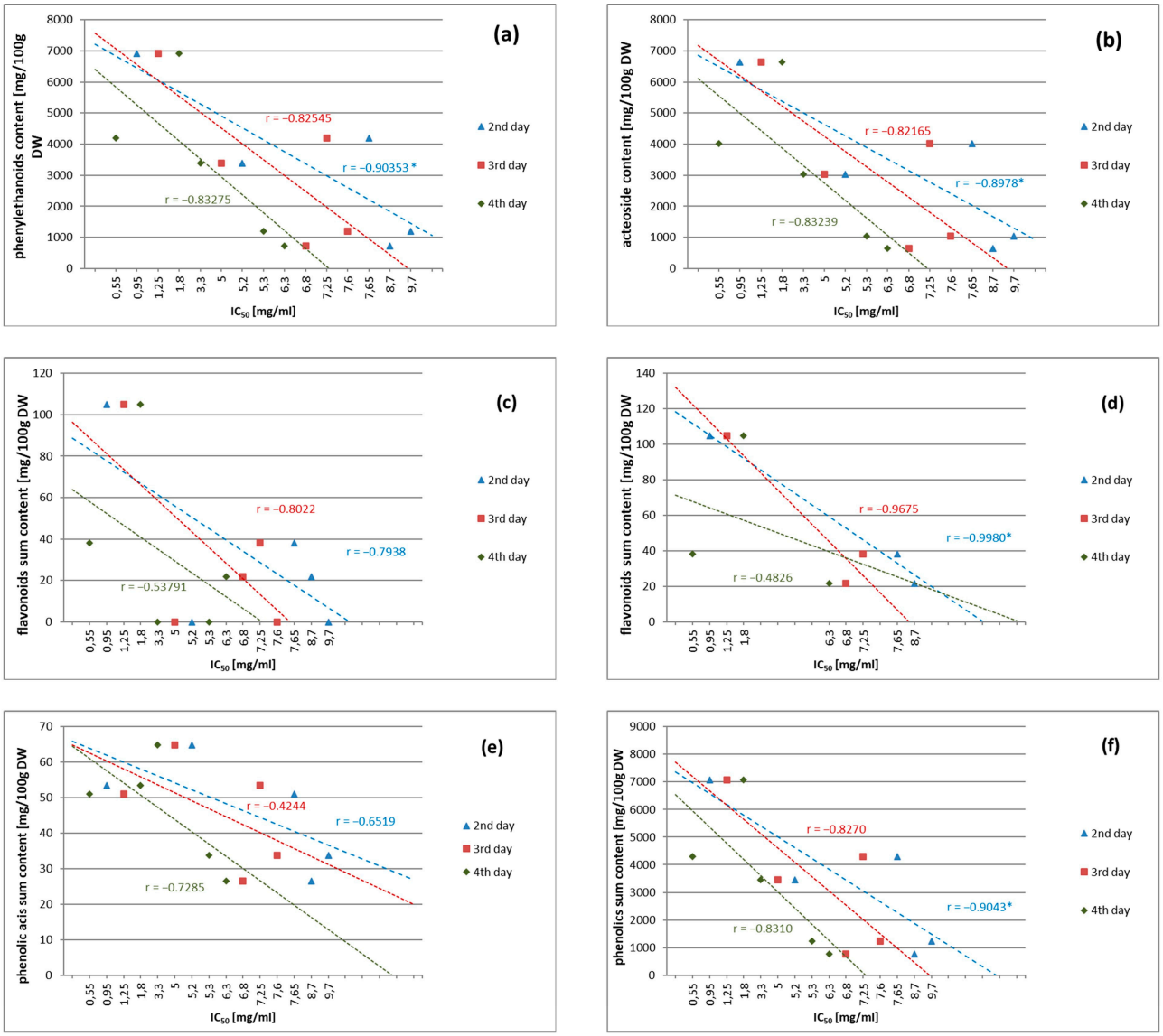


Figure 4. Correlations between IC_{50} values and the content of phenylethanoid glycosides (**a**), acteoside (**b**), phenolic acids (**c**), flavonoids in all materials (**d**), flavonoids without consideration of in vitro-derived materials (**e**), and phenolics sum (phenylethanoid glycosides + phenolic acids + flavonoids) (**f**). * The Pearson correlation coefficients are statistically significant at two-tailed $p < 0.05$.

4. Discussion

Acanthamoeba species have become recognized as dangerous agents leading to diseases including granulomatous amoebic encephalitis (GAE), amoebic keratitis (AK), and amoebic pneumitis (AP). The various synthetic anti-*Acanthamoeba* substances are often ineffective. Regarding the anti-parasitic activity, a plethora of raw materials and groups of bioactive compounds with amoebicidal or amoebistatic properties were studied including abietane diterpenoids from *Salvia sclarea* L. (with the highest activity of ferruginol—72%) [45]; essential oil from *Pterocaulon polystachyum* D.C. [46]; extracts from *Artemisia argyi* H.Lev&Vaniot [47] and *Artemisia annua* L. with the artemisinin as the most active compound [34]; *Lonicera japonica* Thunb. extract with its major constituent chlorogenic acid [48]; *Buddleja cordata* Tunht. extract rich in laminarin belonging to flavones [49]; a brew of green tea (*Camelia sinensis* (L.) Kuntze) rich in flavans [50]; extracts of *Eryngium* species [33,51]; *Rubus chamaemorus* L. extract with ellagic acid as the leading compound; *Pueraria lobata* (Willd.) Ohwi. extract with active isoflavones—puerarin and daidzein [32];

extracts of *Teucrium* species [52]; *Centaurea* species, *Rhaponticum pulchrum* Fisch. et Mey. and *Tanacetum vulgare* L. extracts with sesquiterpene lactones [53]; *Lychnis flos-cuculi* L. extracts with phytoecdysones [54]; and *Chaenomeles japonica* (Thunb.) Lindl. ex Spach extract with a high content of pentacyclic triterpenoids (mainly ursolic, oleanolic, and betulinic acids) [55].

Only amoebicidal substances are useful in the treatment of *Acanthamoeba* infections. Amoebostatic (change of trophozoites into cysts) substances are not suitable for use in therapy. The transformation of amoeba into cysts under the influence of the administered preparation is very unfavorable for the patient. The resulting cysts can survive in the patient's tissues for a very long time and, under conditions favorable for the amoeba, they can cause the invasion to resume. Our task was to find a substance that would kill the trophozoites, but would not cause cyst formation. In the current study, no encystation (transformation of amoeba into cysts) was observed upon application of the tested extracts from *Plantago*.

In the present study, the highest anti-amoebic activity of the water fractions of *Plantago media* was recorded for concentrations of 5 and 10 mg/mL on the third and fourth day of treatment of trophozoites.

Leaf extracts from the crop with the highest content of acteoside had an anti-amoebic effect from the lowest concentration of 1 mg/mL and achieved the lowest IC_{50} value among the tested samples, while extracts from inflorescences and in vitro roots with the lowest content of acteoside also showed the highest IC_{50} index. *Plantago lanceolata* leaf extracts had a lower concentration of acteoside than *P. media* leaves and reached the lowest IC_{50} result, although only on the fourth day of the experiment, which, in turn, may be associated with a different profile of phenolic acids in these species. The presence of flavonoids in the composition does not seem to affect the strength of the antiprotozoal effect, which is confirmed by the particularly good result of this effect of in vitro shoot extracts devoid of these components.

The mechanism behind the antiprotozoal, exactly anti-leishmanial activity of acteoside has been found to be the inhibition of an arginase enzyme, leading to the inhibition of polyamine biosynthesis that is important to infectivity, and has been attributed to the catechol groups in this compound (two adjacent hydroxyl groups on each of the two phenyl rings) [28]. In our studies, the correlation between IC_{50} values and groups of phenolic secondary metabolites was highest for phenylethanoid glycosides and acteoside (R^2 0.6248 and 0.6131, respectively). Although the inhibition of arginase has not been mentioned among modes of anti-acanthamoebic action [30], it is possible due to the ubiquitous occurrence of that enzyme [56]. The brews of green tea (*Camelia sinensis*), known for the high content of catechin derivatives possessing catechol and pyrogallol groups (e.g., EGCG—epigallocatechin 3-gallate), have exhibited pronounced anti-acanthamoebic activity [50]. The authors have hypothesized that the activity could be due to the inhibition of DHFR (dihydrofoliate reductase) and serine protease and metalloprotease enzymes resulting in the disruption of DNA synthesis and cytolysis of *Acanthamoeba* trophozoites, respectively [50].

When it comes to all the *Plantago media* extracts used, it can be seen that the lowest IC_{50}, and thus the highest biological activity, have leaf extracts, both from ground plants (IC_{50} from 0.95 to 1.80 mg/mL) and obtained by biotechnological methods in vitro (3.30 to 5.50 mg/mL). A similar tendency was observed in the case of extracts from the leaves of shoot cultures of *E. campestre* and *E. planum*, for which IC_{50} was estimated to be lower than that for extracts from roots obtained in vitro [51]. As it turned out, this activity did not correlate with the content of polyphenols, which, for example, for *Eryngium planum*, was similar in roots and shoots obtained from micropropagated plants or with the content of triterpene saponins, which was impressively higher in the case of in-vitro-derived roots [55]. If we consider extracts from. *C. japonica*, IC_{50} was the lowest for extracts from leaves of shoots regenerated in vitro than from shoots of plants from natural sites, callus, or fruits [55]. The anti-acanthomoebic activity of the extracts clearly correlated with the content of pentacyclic triterpenoids [55]. Considering the anti-acanthoamoebic activity of *L. flos-cuculi* extracts, 80% methanol fractions from the herb had a higher IC_{50} value than

those from roots or even biotechnologically obtained callus cells [54]. This relationship is probably not conditioned by the amount of main ecdysteroids, because, in the case of this species, the content of the sum of polypodine B and 20-hydroxyecdysone in the herb and roots is similar, while these compounds are not synthesized by callus cells [57]. Among aerial part extracts from plant species belonging to the *Asteraceae* family (*C. bella*, *C. daghestanica*, *R. pulchrum*, and *T. vulgare*), the lowest IC_{50} was calculated for *C. bella*, *C. daghestanica*, and *T. vulgare*, while IC_{50} for *R. pulchrum* was very high (>10 mg/mL). The amoebicidal effect of extracts may be attributed mainly to the sesquiterpene lactones, which are major compounds of four species from *Asteraceae*. The most common sesquiterpene lactones isolated from the investigated plants were guaianolides: cynaropicrin, janerin, and chlorojanerin [53]. On the other hand, the acanthamoebic activity of *Origanum syriacum* and *O. laevigatum* extracts seemed to be very low, even at a concentration of 32 mg/mL [58].

The above-discussed data on the anti-acanthamoebic activity of various plant species are compiled in the Table 5.

Table 5. IC_{50} value for the studied discussed plant species.

Plant Species	IC_{50} on 2nd Day	References
Plantago media L.	Water-soluble fr of MeOH extract from leaves of intact plants, 0.95 mg/mL Water-soluble fr of MeOH extract leaves of in vitro plants, 5.20 mg/mL	This work
Salvia sclarea L.	Ferruginol 0.05 mg/mL Salvipisone 0.4 mg/mL	[45]
Pterocaulon polystachyum DC	Essential oil 10 mg/mL	[46]
Artemisia argyi Levl. et Vant.	MeOH extract 37.4 mg/mL (8 h)	[47]
Artemisia annua L.	MeOH extract 12.5 mg/mL Artemisinin 0.12 mg/mL	[34]
Buddleya cordata H.B.K.	N.R. amoebostatic at 32 mg/mL	[49]
Camelia sinensis (L.) Kuntze	N.R. 50% brew	[50]
Eryngium planum L.	N.R. 50% inh. 5 mg/mL	[33,51]
Rubus chamaemorus L.	MeOH extract > 0.05 mg/mL	[32]
Pueraria lobata (Willd.)	MeOH extract > 0.01 mg/mL	[32]
Solidago virgaurea L.	MeOH extract > 0.01 mg/mL	[32]
Solidago graminifolia Elliott.	MeOH extract > 0.05 mg/mL	[32]
Teucrium polium L.	MeOH extract 60% inh. 16 mg/mL	[52]
Teucrium chamaedrys L.	MeOH extract 53% inh. 8 mg/mL	[52]
Lychnis flos-cuculi L.	40% fr MeOH herb extract > 1.0 mg/mL 80% fr MeOH herb extract > 1.0 mg/mL 40% fr MeOH root extract > 5.0 mg/mL 80% fr MeOH root extract > 0.5 mg/mL 20-hydroxyecdysone > 0.5 mg/mL Polypodine B > 0.5 mg/mL	[54]
Chaenomeles japonica L.	EtOH fruit extract 6.25 mg/mL EtOH leaves extract 1.80 mg/mL EtOH leaves (in vitro) extract 0.3 mg/mL EtOH callus extract 2.30 mg/mL	[55]
Centaurea bella Trautv.	EtOH extract 0.61 mg/mL	[53]
Centaurea daghestanica Sosn.	EtOH extract 0.86 mg/mL	[53]

Table 5. *Cont.*

Plant Species	IC_{50} on 2nd Day	References
Rhaponticum pulchrum Fisch. Et Mey	EtOH extract > 10 mg/mL	[53]
Tanacetum vulgare L.	EtOH extract 0.64 mg/mL	[53]

IC_{50}—the half-maximum anti-amoebic inhibitory concentration; N.R.—not reported; and Inh.—inhibition.

5. Conclusions

Our investigations show that *Plantago media*, either from in vitro cultures or soil cultivation, exerts an interesting effect against protozoan parasites such as *Acanthamoeba* sp. trophozoites in a manner dependent on the content of the main phenolic component, a phenylethanoid glycoside—acteoside (verbascoside). These findings imply that further investigations are needed to substantiate the role of acteoside, and the structurally related phenylethanoid glycosides, in the anti-acanthamoeba activity.

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