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Evaluation of Antiamoebic and Antimicrobial Activities *In Vitro* of *Chaenomeles japonica* (Thunb.) Lindl. ex Spach Extracts

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The free-living *Acanthamoeba* sp. causes various diseases. Treatment of them is very difficult and not always effective because of encystation, making it highly resistant to antiamoebic drugs. Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli*, and an yeast *Candida albicans* also exhibit outstanding resistance to antimicrobial substances. The search for new natural amoebicidal and antimicrobial agents of plant origin is still of current interest. The aim of the study was to investigate the amoebicidal activity of the extracts obtained from tissue culture and a field-grown plant of *Chaenomeles japonica* against pathogenic trophozoites of *Acanthamoeba* spp. and antimicrobial effect against *S. aureus*, *E. coli*, and *C. albicans*. The extracts of *C. japonica* had an inhibitory effect on the proliferation of *Acanthamoeba* trophozoites as compared to the non-treated control. Among the crude extracts tested, the extract of leaves, from both shoot culture and the field-grown plant had remarkable amoebicidal action against the trophozoites but also antibacterial activity against Gram-positive bacteria *Staphylococcus aureus*. The extract from leaves from shoot culture, already on the second and third days of treatment, showed an antiamoebicidal effect at a concentration of 1 mg mL^{-1} (inhibition of trophozoites 87.5% and 91.8%, respectively). In addition to leaves from shoot culture (a conc. 5 mg mL^{-1}, 2nd day inhibition of trophozoites 85.7% and 3rd day 97.2%), leaves from a field-grown plant (a conc. 5 mg mL^{-1}, 2nd day 91.0% and 3rd day 94.4%) and callus (a conc. 5 mg mL^{-1}, 2nd day 90.0% and 3rd day – 95.4%) also exhibited a good antiamoebicidal activity. Out of the four extracts, the extracts from leaves from both shoot culture and a field-grown plant were reported to be the most active against Gram-positive *S. aureus*, which was determined by the values of MIC = 5.0 mg mL^{-1} and MIC = 2.5 mg mL^{-1}, respectively. The inhibitory potential depends on the yield and composition of mainly bioactive compounds: pentacyclic terpenoids (mainly betulinic, ursolic, and oleanolic acids) and polyphenols (mainly chlorogenic acid and its isomers, epicatechin, dimeric, and trimeric proanthocyanidins, quercetin and kaempferol derivatives).

Keywords: *Acanthamoeba* spp., *Chaenomeles japonica*, pentacyclic terpenoids, plant extracts, plant *in vitro* cultures, polyphenols, *Staphylococcus aureus*

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INTRODUCTION

In the literature, there is more scientific information on the plant extracts with amoebicidal activity against pathogenic strains of *Acanthamoeba* spp. (Derda et al., 2009; Derda et al., 2016; Hadaś et al., 2017a; Hadaś et al., 2017b; Tanveer et al., 2018). Those free-living protozoan saprophytic organisms naturally occurring in the environment show strong tendencies to change their lifestyle to parasitic. *Acanthamoeba* infections are observed during all the seasons and are usually linked to contact with water and moist soil. In recent years, there have been an increasing number of patients with weakened immune systems, susceptible to infections and contagions, including infestations caused by protozoa of the genus *Acanthamoeba*. The group of special risk for infections caused by potentially pathogenic protozoa includes adults with acquired immunodeficiency syndrome using contact lenses, and who, at the same time, are physically active in the natural environment. The organisms occur in two forms, namely trophozoites, which can be invasive to humans, and cysts as spore forms. They may occur in air, water, soil, fresh and saltwater reservoirs, swimming pools, air conditioning, sanitary facilities, and tap water. They were also detected in bottled spring water and contact lens fluids (Hadaś et al., 2017a; Padzik et al., 2017). These protozoans may be the cause of amoebic keratitis as well as chronic granulomatous amoebic encephalitis, pneumonia, and lesions in other human organs. The treatment of acanthamoebosis is difficult and not always effective so antibiotics and highly irritating disinfectants are applied (Derda et al., 2009; Niyyati et al., 2016). Due to the problems in treatment and lack of effective but safe drugs, the search continues for substances of plant origin that, applied as combined therapy, could contribute to decreasing of the effective concentrations of the used antibiotics (Derda et al., 2013; Niyyati et al., 2016; Padzik et al., 2017). Nowadays, the use of plant crude extracts and isolated bioactive compounds is increasing also due to growing resistance of microbes against antibiotics and antifungal agents. Numerous studies have been conducted on the antimicrobial activity of medicinal plants – screening for their activity towards Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli*, and an opportunistic yeast *Candida albicans* (Vashist and Jindal, 2012; Kikowska et al., 2016).
Chaenomeles japonica (Thunb.) Lindl. ex Spach (Japanese quince, Pomoideae, Rosaceae), a plant species with a global distribution, is a source of edible aromatic fruits containing several bioactive compounds that make *C. japonica* a potentially effective medicinal plant (Nahorska et al., 2014). Phytochemical studies have yielded a various level of secondary metabolites in other raw materials, namely pentacyclic triterpenoids – ursolic, oleanolic, betulinic, and pomolic acids (Xu et al., 2002; Du et al., 2013; Kikowska et al., 2018, 2019), flavonoids – flavonols, mostly quercetin, and kaempferol derivatives, and epicatechin (Xu et al., 2002; Du et al., 2013; Kikowska et al., 2018, 2019), phenolic acid – caffeic acid (Sokołowska-Woźniak et al., 2002, Kikowska et al., 2018), dicaffeoylquinic acid (Kikowska et al., 2018), leucoanthocyanin, monoterpene glucosides, roseoside, steroid saponin (daucosterol), and prunasin (Xu et al., 2002). Due to its composition and antioxidant properties, Japanese quince is considered to be a potential source of valuable compounds for medicinal and cosmetic uses (Xu et al., 2002; Nahorska et al., 2014; Kikowska et al., 2018, 2019).

There is a growing interest in pentacyclic triterpenoids because of their wide potential of biological and pharmaceutical properties. Oleanolic acid and its isomer – ursolic acid, have long been known to be anti-inflammatory, hepatoprotective, and anti-hyperlipidemic in traditional medicine of Asia. Moreover, recently there have been many studies on their antiviral, antimicrobial, and anticancer activities (Babalola and Shode, 2013). Betulinic acid is highly regarded for its anti-HIV-1 activity and cytotoxicity against cancer lines (Yogeeswari and Sriram, 2005; Moghaddam et al., 2012). Polyphenols are the most common bioactive compounds widely distributed in plants and they exhibit a great diversity. The phenolic constituents exhibit diversified biological activity, mainly attributed to their antioxidant potential (Rasouli et al., 2017). The main phenolic acid occurring in *C. japonica* is chlorogenic acid, which is a well-known polyphenol, exerting many biological activities, i.e., antioxidant, antimicrobial, antipyretic, hepatoprotective, cardioprotective, anti-inflammatory, anticarcinogenic, and acts as a modulator of glucose and lipid metabolism (Nabeed et al., 2018).

The aim of the present study was to investigate the amoebicidal or amoebistatic *in vitro* effect of *Chaenomeles japonica* extracts, obtained from *in vitro* cultures – callus (tissue culture) and leaves from shoot culture as well as leaves and fruits from field-grown plant, on the growth and development of free-living trophozoites of *Acanthamoeba* spp. Moreover, the studied extracts were evaluated *in vitro* for antimicrobial effect against *Staphylococcus aureus*, *Escherichia coli*, and *Candida albicans*. *C. japonica* extracts have not yet been studied for antiamoebic and antimicrobial activities.

MATERIALS AND METHODS

PLANT MATERIAL

A voucher specimen of *C. japonica* (no. 1526/2016) was deposited in the Herbarium of Department of Pharmaceutical Botany and Plant Biotechnology, Poznań, Poland. The fruits and leaves were collected from a field-grown old shrub (52°21′55.4″ N 17°00′12.5″ E; 52.365381, 17.003471) in Poznań, in 2013. The aseptic *in vitro* culture initiation, shoots multiplication (shoot culture), as well as tissue culture (callus) induction and proliferation (Fig. 1) were performed according to Kikowska et al. Callus culture was initiated from leaves of the micropropagated plantlet explants and stabilized on Murashige and Skoog (MS) media with 2.0 mg L^{-1} dichlorophenoxyacetic acid and 0.2 mg L^{-1} 1-naphtaleneacetic acid (passage 25th). The shoots were multiplied on MS media enriched with 1.0 mg L^{-1} N6-benzyladenine and 1.0 mg L^{-1} indole-3-acetic acid every six weeks of subculture (Kikowska et al., 2018).

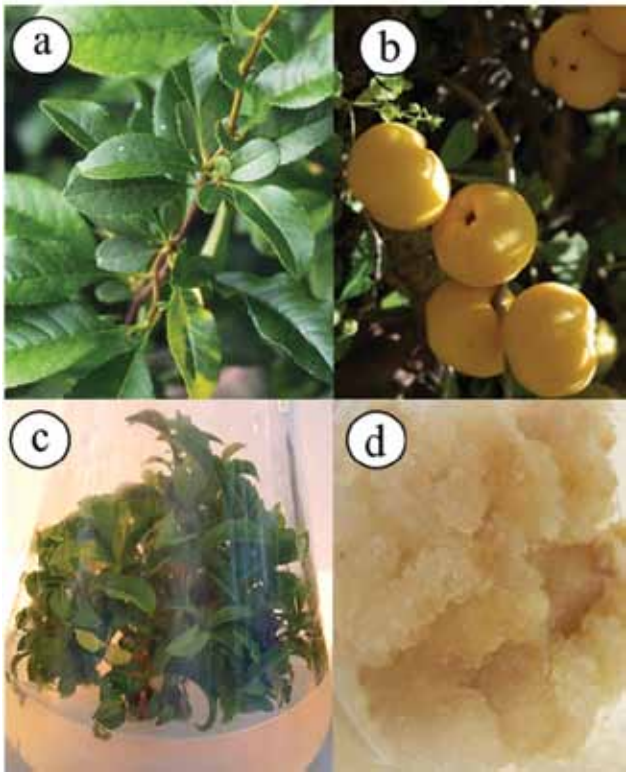


Fig. 1. *Chaenomeles japonica* (**a**) leaves from field-grown plant, (**b**) fruits, (**c**) leaves from shoot culture on MS media with 1.0 mg L^{-1} BAP and 1.0 mg L^{-1} IAA, (**d**) callus on MS with 2.0 mg L^{-1} 2,4D + 0.2 mg L^{-1} NAA.

PREPARATION OF PLANT EXTRACTS

The lyophilized parts of plants (leaves and fruits of a field-grown plant) and biomass from *in vitro* cultures (callus and leaves from shoot cultures) were extracted three times with EtOH 70% (v/v) at 95°C. The extracts were concentrated under reduced pressure and used for further studies, i.e., evaluation of antiamoebic and preliminary antimicrobial studies.

PHYTOCHEMICAL SCREENING

The phytochemical analysis of *C. japonica* extracts was performed according to Kikowska et al. (2018). The samples (100 mg) were extracted with 80% (v/v) methanol using ASE 200 system (100°C, 10.3 MPa) in three extraction cycles, the total extraction time was 27 min, the final extract volume was 24 mL. The extracts were subsequently evaporated to dryness. The dry extracts were suspended in 5% MeOH and subjected to solid phase extraction on Waters SepPak cartridge. The analytes were eluted with 95% MeOH, evaporated to dryness, and reconstituted in 3 mL of 90% MeOH. Before the analysis, the samples were centrifuged (23000 × g, 15 min). When necessary (determination of triterpenoid acids; determination of chlorogenic acid in leaves from the field-grown plant), samples were 10-fold diluted. UHPLC-DAD-ESI-MS analyses were performed using ACQUITY UPLC® chromatographic system (Waters, Milford, MA, USA) with a triple quadrupole mass detector. The qualitative analyses of *C. japonica* extracts and also the quantitative analyses of chlorogenic acid and other phenolics, were performed employing ACQUITY BEH C18 column (2.1 × 100 mm, 130 Å, 1.7 μm; Waters) at 50°C; the flow rate was 0.400 mL min^{-1}, and the injection volume 2.5 μL. Gradient elution was applied, using 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) as mobile phase constituents. Constituents of the investigated extracts were tentatively identified and classified on the basis of their UV spectra and/or MS data. Chlorogenic acid and other phenolics were quantified or semi-quantified on the basis of UV chromatograms, using a calibration curve of chlorogenic acid (Sigma-Aldrich). The content of ursolic, oleanolic acid, and the putative betulinic acid in the investigated samples was determined using ACQUITY HSS C18 column (2.1 × 100 mm, 100 Å, 1.8 μm; Waters); the flow rate was 0.400 mL min^{-1} (30°C and the injection volume was 2.5 μL. The analytes were separated isocratically for 11.9 min using 80% methanol containing 0.1% formic acid. Detection of triterpenoid acids was performed using the

Selected Ion Monitoring (SIM) method. The content of ursolic and oleanolic acids was calculated using calibration curves of authentic standards. Betulinic acid content was expressed as the oleanolic acid equivalent. More details of chromatographic conditions, mass spectrometer settings, and quantitation methods can be found in the work of Kikowska et al. (2018). The extractions and analyses were performed in triplicate; the presented results are means with the standard deviation.

PARASITOLOGICAL EXAMINATION

In this study we used the *Acanthamoeba* sp. strain 309 deposited in GenBank (NCBI) under the accession number KY203908, pathogenic for mice and isolated from the environment [19]. The amoebae were axenically cultured on the liquid medium containing 2% Bacto-Casitone and 10% horse serum.

Parasitological examination of extracts was performed according to Derda et al. (2009). The study investigated the activity of ethanol extracts from *in vitro* cultures, including callus and leaves from shoot cultures and the extracts from leaves and fruits from the field-grown plant. The increase in the number of trophozoites was examined at 24-hour intervals, using Thoma haemocytometric chamber for counting cells. The control consisted of cultured trophozoites without any extracts. The relationship between the extract concentration and the time of treatment of trophozoit cultures was investigated.

PRELIMINARY ANTIMICROBIAL ACTIVITY EXAMINATION

Antimicrobial activity of extracts was assessed by determination of minimal inhibitory concentration (MIC) and minimal biocidal concentration (MBC). The extracted plant material was suspended in DMSO (final conc. 100 mg mL^{-1}). The bacteria strains (Gram-positive *Staphylococcus aureus* ATCC 25923 and Gram-negative *Escherichia coli* ATCC 25922) were cultured in the brain-heart infusion broth (BHI), while *Candida albicans* ATCC 27853 was cultured in Sabouraud dextrose broth (SDB) at 36 ± 1°C for 20 h. Antimicrobial activity of the examined extracts was studied by employing a broth microdilution method in accordance with EUCAST (European Committee on Antimicrobial Susceptibility Testing) guidelines with some modifications. Aliquots of 150 μL of each dilution were distributed in 96-well plates. The test wells were inoculated with 150 μL of a standardized suspension of test microorganisms. The final bacteria concentration was about 5 × 105 CFU mL^{-1} and fungus about 2.5 × 105 CFU mL^{-1}. The sterility control (a diluted extract) and a growth control (culture broth with microbial suspension) were sampled in the same manner. The plates were incubated at 34°C for 18 h. The MIC was defined as the lowest concentration at which no visible growth was observed. To determine MBC, the samples at a concentration equal to and greater than the MIC were subcultured onto the agar medium: Typcase soy agar (TSA) – bacteria; SDA – fungus. The plates were incubated at 34°C for 18 h; the MBC was defined as the lowest concentration at which no growth was observed.

STATISTICAL ANALYSIS

The mean number (MN) of amebae and the standard deviation (SD) were calculated in each measurement group. The statistical analysis was determined applying the Mann-Whitney and ANOVA tests. Statistical significance was defined as $P < 0.05$.

RESULTS

The results of the study indicate that all the extracts obtained from Japanese quince, both from *in vitro* cultures (callus and leaves from shoot cultures) and from organs of the field-grown plant (leaves and fruits), inhibited the growth of *Acanthamoeba* sp. trophozoites to different degrees (Tables 1–4; Fig. 2). The dependence of the effect on the extract concentration and treatment time was noted. The strongest effect was observed for leaves from *in vitro* shoot culture (Table 2, Fig. 2) and for leaves of the field-grown plant (Table 3, Fig. 2). The extract from leaves from shoot culture, already on the second and third days of treatment, showed an antiamoebicidal effect at a concentration of 1 mg mL^{-1}. The other extracts at a dose of 1 mg mL^{-1} weakly inhibited the development of trophozoites. In addition to leaves from shoot culture and leaves from the field-grown plant, callus also exhibited antiamoebicidal activity at a concentration of 5 mg mL^{-1} after the second and third days of treatment. After the third day of treatment, this extract inhibited the growth of trophozoites by 95%. The callus extract was the strongest on the third day after applying the concentration of 10 mg mL^{-1}, inhibiting the growth of amphibians by 99.2%. At this concentration it also exhibited a strong effect on the second day (92.5%). The results indicate that the ethanol-aqueous extracts from callus act depending on the concentration and time of treatment of the trophozoit cultures. Their effects on *Acanthamoeba* sp. trophozoites were demonstrated, mainly at 5 and 10 mg mL^{-1} (Table 1, Fig. 2). The fruit extract had a very poor effect (Table 4, Fig. 2).

The highest IC_{50} index was calculated for leaves from shoot culture extract (Table 5). On the second and third days of treatment, the IC_{50} value was 0.30 mg mL^{-1}. The leaf extract from the field-grown plant also showed strong antiamoebicidal activity, however, 3–4 times weaker than leaves from shoot culture. For this extract, the IC_{50} value on the second day was 1.80 mg mL^{-1} and on the third 1.10 mg mL^{-1}. The callus extract had a low ratio – on the second day the IC_{50} was 2.30 mg mL^{-1} and on the third IC_{50} was 2.75 mg mL^{-1}. The extract from fruits exhibited the weakest effect among the tested extracts as on the second day the IC_{50} value was 6.25 mg mL^{-1} (Table 5).

Out of the four extracts, the extracts from leaves from both shoot culture and the field-grown plant were reported to be the most active against Gram-positive *Staphylococcus aureus*, which was determined by the values of MIC = 5.0 mg mL^{-1} and MIC = 2.5 mg mL^{-1}, respectively. As it results from the presented study, moderate activity against this bacterium was shown for the extract from fruits (MIC = 10.0 mg mL^{-1}), while the weakest activity was exhibited by the extract from callus (MIC = 20.0 mg mL^{-1}). The tested extracts, apart from the callus extract, were found to show similar activity against Gram-negative *Escherichia coli*, with MIC = 40.0 mg mL^{-1}. The extract from leaves from shoot culture, among all the tested extracts, was reported to have the best antifungal effect (Table 7).

The phytochemical screening revealed the presence of phenolic compounds (mainly chlorogenic acid and its isomers, epicatechin, dimeric, and trimeric proanthocyanidins) and triterpenoids (among them the uncharacterized ones, namely well-known betulinic, ursolic, and oleanolic acids) in the studied extracts. Moreover, the presence of quercetin and kaempferol derivatives was detected in the leaves from shoot

TABLE 1. The effect of callus extract of *Chaenomeles japonica* on inhibition of *Acanthamoeba* trophozoites during three-day treatment.

Extract conc. [mg mL^{-1}]	Day treatment					
	1st		2nd		3rd	
	MN ± SD	GI [%]	MN ± SD	GI [%]	MN ± SD	GI [%]
Control	7.85 ± 3.44	0	26.73 ± 6.27	0	41.75 ± 5.98	0
1	7.08 ± 3.33	9.81	21.05 ± 3.2	21.25	39.17 ± 11.9	6.18
5	7.33 ± 3.01	6.63	2.67 ± 2.53*	90.02	1.92 ± 3.04*	95.41
10	6.16 ± 3.86	21.53	2.00 ± 2.58*	92.52	0.33 ± 0.62*	99.21

MN – mean number of trophozoites; **GI** – growth inhibition

* $P < 0.05$ statistically significant differences from control during the same time interval; n = 18

TABLE 2. The effect of leaves from shoot culture extract of *Chaenomeles japonica* on inhibition of *Acanthamoeba* trophozoites during three-day treatment.

Extract conc. [mg mL^{-1}]	Day treatment					
	1st		2nd		3rd	
	MN ± SD	GI [%]	MN ± SD	GI [%]	MN ± SD	GI [%]
Control	7.85 ± 3.44	0	26.73 ± 6.27	0	41.75 ± 5.98	0
1	5.92 ± 2.90	24.59	3.33 ± 2.89*	87.55	3.42 ± 1.50*	91.81
5	4.17 ± 1.95	46.88	3.83 ± 1.34*	85.68	1.17 ± 1.34*	97.20
10	3.92 ± 2.25	50.07	2.17 ± 1.14*	91.89	1.33 ± 0.94*	96.82

MN – mean number of trophozoites; **GI** – growth inhibition

* $P < 0.05$ statistically significant differences from control during the same time interval; n = 18

TABLE 3. The effect of leaves from field-grown plant extract of *Chaenomeles japonica* on inhibition of *Acanthamoeba* trophozoites during three-day treatment.

Extract conc. [mg mL^{-1}]	Day treatment					
	1st		2nd		3rd	
	MN ± SD	GI [%]	MN ± SD	GI [%]	MN ± SD	GI [%]
Control	7.85 ± 3.44	0	26.73 ± 6.27	0	41.75 ± 5.98	0
1	4.75 ± 1.95	39.50	17.36 ± 7.47	35.06	21.75 ± 4.73*	47.91
5	3.30 ± 3.46	57.97	2.42 ± 1.89*	90.95	2.33 ± 2.29*	94.42
10	2.67 ± 1.83	65.99	2.08 ± 1.71*	92.22	1.67 ± 1.31*	96.00

MN – mean number of trophozoites; **GI** – growth inhibition
* $P < 0.05$ statistically significant differences from control during the same time interval; n = 18

TABLE 4. The effect of fruit extract of *Chaenomeles japonica* on inhibition of *Acanthamoeba* trophozoites during three-day treatment.

Extract conc. [mg mL^{-1}]	Day treatment					
	1st		2nd		3rd	
	MN ± SD	GI [%]	MN ± SD	GI [%]	MN ± SD	GI [%]
Control	7.85 ± 3.44	0	26.73 ± 6.27	0	41.75 ± 5.98	0
1	7.45 ± 2.74	5.10	17.67 ± 3.77	33.90	41.17 ± 7.29	1.39
5	6.5 ± 1.71	17.20	15.92 ± 6.06	40.45	41.25 ± 8.92	1.20
10	2.75 ± 1.36*	64.97	4.00 ± 1.35*	85.04	21.42 ± 7.10*	48.70

MN – mean number of trophozoites; GI – growth inhibition
*$P < 0.05$ statistically significant differences from control during the same time interval; n = 18

culture and the field-grown plant as well as naringenin hexoside in the leaves from the field-grown plant (Fig. 3). Leaves of *Ch. japonica* are a rich source of diverse phenolic compounds, especially, chlorogenic acids. However, phenolic profiles of field grown and shoot culture plants differ significantly. Leaves of the field grown plant are very rich in chlorogenic acid, they also contain much smaller amounts of a chlorogenic acid isomer, as well as flavonoids (quercetin hexoside-deoxyhexoside, kaempferol hexoside-deoxyhexoside, quercetin hexoside, eriodictyol hexoside, kaempferol hexoside, naringenin hexoside), a dicaffeoylquinic acid, as well as caffeic acid and *p*-coumaric acid hexose derivatives. In contrast, major phenolics of leaves from the shoot culture plants are chlorogenic acid (much lower level than in field plants) and a dicaffeolyquinic acid. Two isomers of chlorogenic acid, three other dicaffeoylquinic acids, flavonoids (kaempferol hexoside-deoxyhexoside, quercetin hexoside, kaempferol deoxyhexoside-deoxyhexoside, kaempferol hexoside), epicatechin, and a coumaric acid hexose derivative were also present. Phenolic composition of the fruit and the callus was much less complex. They contained mainly chlorogenic acid, different proanthocyanidins, epicatechin and quercetin hexoside (in the callus only). Chlorogenic acid content was much lower than in the natural and *in vitro* leaves. As regards triterpenoids, oleanolic acid, ursolic acid and the putative betulinic acid were present in all investigated samples. In addition, a number of putative triterpenoids, giving deprotonated ions at *m/z* 469, *m/z* 471, *m/z* 473, *m/z* 485 and *m/z* 487 were also detected.

The best antimicrobial and antiamoebicidal effect of leaves, from both shoot culture and the field-grown plant, may be also explained by the highest content of the pentacyclic triterpenoids (mainly ursolic, oleanolic, and betulinic acids) in the studied extracts, i.e., 5.010 and

Fig. 2. Effect of *Chaenomeles japonica* (**a**) callus, (**b**) leaves from shoot culture, (**c**) leaves from field-grown plant and (**d**) fruit extracts [1 mg mL^{-1}, 5 mg mL^{-1}, 10 mg mL^{-1}] on inhibition of *Acanthamoeba trophozoites* proliferation in culture medium.

TABLE 5. Determination of IC_{50} [mg mL^{-1}] for the studied extracts of *Chaenomeles japonica*.

Plant material	IC_{50} 1st day	IC_{50} 2nd day	IC_{50} 3rd day
Callus	–	2.30	2.75
Leaves from shoot culture	7.00	0.30	0.30
Leaves from field-grown plants	1.90	1.80	1.10
Fruits	8.50	6.25	–

IC_{50} – the half maximum antiamoebic inhibitory concentration

5.363 mg g^{-1} d.w., respectively, which is about 1.3–1.4 more than in the fruit extract and 1.9–2.0 than in the callus extract (Table 8). Also, there is the highest content of the sum of polyphenols (Table 8). Chlorogenic acid, the main phenolic acid, occurs in the extract from leaves of the field-grown plant at a concentration about 15 times higher than in the callus extract and 21 times higher than in the fruit extract, and in leaves from shoot culture 3.4 times higher than in the callus extract and 4.9 than in the fruit extract (Table 8); those compounds may act synergically.

DISCUSSION

Currently, there is an increasing interest in plant therapies that can be effective in the treatment of chronic diseases. In many cases, substances of plant origin and plant preparations successfully supplement conventional treatment of several diseases without causing any side effects. They can be used in treatment of, for example, parasitological diseases or in the cases of infection caused by microorganisms (Derda and Hadaś, 2015; Niyyati et al., 2016). For a number of plant extracts and isolated compounds, antiprotozoal activity, including activity against pathogenic *Acanthamoeba* strains (Chu et al., 1998; Kayser et al., 2003; Derda et al., 2009; Kuźma et al., 2015; Niyyati et al., 2016; Hadaś et al., 2017a; Hadaś et al., 2017b) and antimicrobial activity (De Leon et al., 2005; Chung et al., 2011; Vashit and Jindal, 2012; EL-Zawahry et al., 2013; Kikowska et

TABLE 6. Inhibition [%] of trophozoites from the *Acanthamoeba* on three consecutive days of treatment with *Chaenomeles japonica* extracts.

Plant material	Extract concentration											
	Control			1 mg mL^{-1}			5 mg mL^{-1}			10 mg mL^{-1}		
	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd	1st	2nd	3rd
Callus	0	0	0	9.8	21.2	6.2	6.63	90.0	95.4	21.5	92.5	99.2
Leaves from shoot culture	0	0	0	24.6	875.	91.8	46.9	85.7	97.2	50.1	91.9	96.8
Leaves from field-grown plant	0	0	0	39.5	35.0	47.9	58.0	91.0	94.4	66.0	92.2	96.0
Fruits	0	0	0	5.1	34.0	1.4	17.2	40.4	1.2	65.0	85.0	48.7

TABLE 7. Determination of MIC and MBC/MFC [mg mL^{-1}] of *Chaenomeles japonica* extracts against different microorganisms.

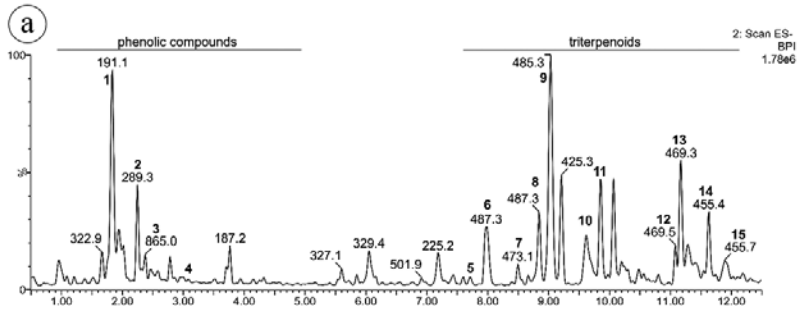
Microorganism	Callus		Leaves from shoot culture		Leaves from field-grown plant		Fruits	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
S. aureus	20.0	160.0	5.0	40.0	2.5	10.0	10.0	40.0
E. coli	–	160.0	40.0	80.0	40.0	40.0	40.0	80.0
C. albicans	–	80.0	10.0	80.0	40.0	160.0	–	160.0

MIC – Minimal Inhibitory Concentration, **MBC** – Minimal Biocidal Concentration

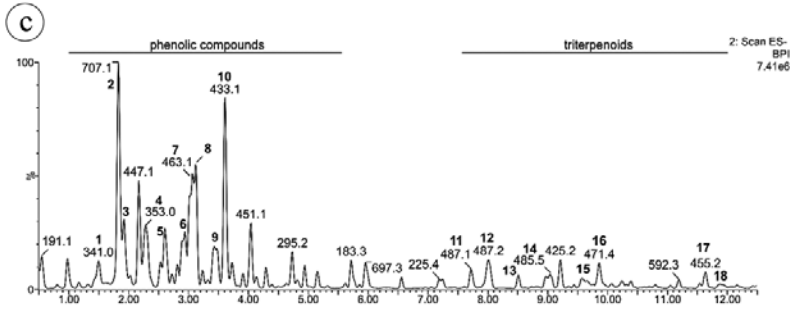
al., 2016; Cheesman et al., 2017) has been demonstrated.

Pentacyclic triterpenoids are known to exert an antimicrobial effect by physically destroying the integrity of biological membranes (Parades et al., 2011). The extracts from olive leaves – *Olea europaea*, a species used in the cosmetics industry, were tested for their activity against *Acanthamoeba* trophozoites. Inhibition of proliferation of *Acanthamoeba* trophozoites after the treatment with triterpene acids – oleanolic acid as well as with butyric acid was shown (Sifaouri et al., 2014). In another work, antiprotozoal activity of studied extracts rich in several compounds, including ursolic acid, was demonstrated (Sifaouri et al., 2017). It was found that ursolic acid induces apoptosis in the process of programmed cell death in treated *Acanthamoeba* sp. trophozoites through the mitochondrial pathway. Antiprotozoal activity of other pentacyclic triterpenoids has been previously reported. Maslinic acid, a pentacyclic derivative present in the olive fruit (*Olea europaea*), is capable of blocking the entry of *Toxoplasma gondii* tachyzoites into the cell and can inhibit some of its proteases. It also produces gliding motility and ultrastructure alterations in parasites (De Pablos et al., 2010). Additionally, antiprotozoal activity of oleanolic acid and its isomer – ursolic acid, has been documented in *Plasmodium falciparum*, *Leishmania donovani*, *L. major*, *L. amazonensis*, *L. infantum*, and *T. cruzi* (Steele et al., 1999; Tan et al., 2002; van Baren et al., 2006; Cunha et al., 2006; AlMusayeib et al., 2013; Simelane et al., 2014; Gnoatto et al., 2018).

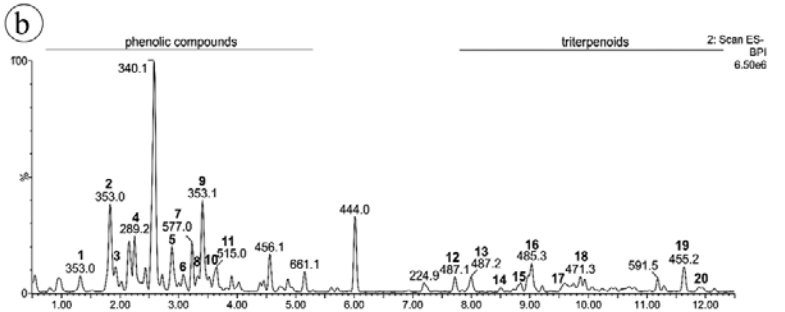
Derda et al. (2013) reported an amoebicidal effect of the phenolic acid fraction from *Eryngium planum* against pathogenic *Acanthamoeba* trophozoites. The effect of inhibition of the growth of *A. triangularis* trophozoites was observed for the fraction of ethyl acetate extract from flower buds of *Lonicera japonica* Thunb. and the main compound, i.e., chlorogenic acid (Moghaddam et al., 2012). In the light of the literature on the biological action of pentacyclic triterpenes (Moghaddam et al., 2012; Babalola and Shode, 2013; Niyyati et al., 2016) and phenolic acids



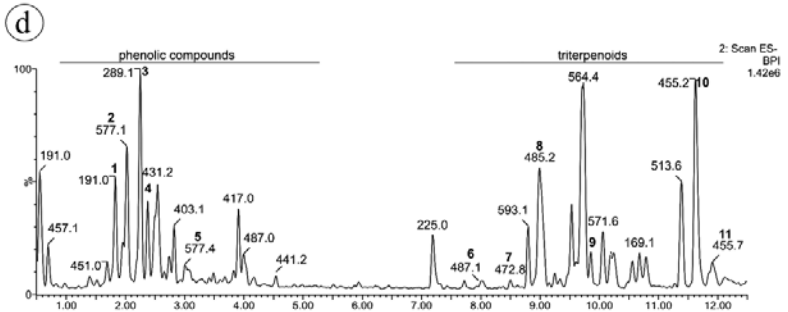
1 – chlorogenic acid; **2** – epicatechin; **3** – trimeric proanthocyanidin; **4** – quercetin hexoside; **5** – a triterpenoid (*m/z* 487); **6** – a triterpenoid (*m/z* 487); **7** – a triterpenoid (*m/z* 473); **8** – a triterpenoid (*m/z* 487); **9** – a triterpenoid (*m/z* 485); **10** – a triterpenoid (*m/z* 471); **11** – a triterpenoid (*m/z* 471); **12** – a triterpenoid (*m/z* 469); **13** – a triterpenoid (*m/z* 469); **14** – putative betulinic acid (*m/z* 455); **15** – ursolic & oleanolic acid (*m/z* 455).



1 – caffeic acid-hexose; **2** – chlorogenic acid; **3** – p-coumaric acid-hexose; **4** – a chlorogenic acid isomer; **5** – quercetin-hexose-deoxyhexose; **6** – kaempferol-hexose-deoxyhexose; **7** – quercetine hexoside; **8** – eriodictyol hexoside; **9** – a dicaffeoylquinic acid & kaempferol hexoside; **10** – naringenin hexoside; **11** – a triterpenoid (m/z 487); **12** – a triterpenoid (m/z 487); **13** – a triterpenoid (m/z 473); **14** – a triterpenoid (m/z 485); **15** & **16** – a triterpenoid (m/z 471); **17** – putative betulinic acid (m/z 455); **18** – ursolic & oleanolic acid (m/z 455)



1 – an isomer of chlorogenic acid; **2** – chlorogenic acid; **3** – an isomer of chlorogenic acid & p-coumaric acid-hexose (traces); **4** – epicatechin; **5** – kaempferol-hexose-deoxyhexose; **6** – quercetin hexoside; **7** – kaempferol dideoxyhexoside; **8** – dicaffeoylquinic acid; **9** – dicaffeoylquinic acid isomer & kaempferol hexoside; **10** – dicaffeoylquinic acid isomer; **11** – dicaffeoylquinic acid; **12** – a triterpenoid (m/z 487); **13** – a triterpenoid (m/z 487); **14** – a triterpenoid (m/z 473); **15** – a triterpenoid (m/z 487); **16** – a triterpenoid (m/z 485); **17** & **18** – a triterpenoid (m/z 471); **19** – putative betulinic acid (m/z 455); **20** – ursolic & oleanolic acid (m/z 455).



1 – chlorogenic acid; **2** – a dimeric proanthocyanidin (*m/z* 577); **3** – epicatechin; **4** – a trimeric proanthocyanidin (*m/z* 865); **5** – a dimeric proanthocyanidin (*m/z* 577); **6** – a triterpenoid (*m/z* 487); **7** – a triterpenoid (*m/z* 473); **8** – a triterpenoid (*m/z* 485); **9** – a triterpenoid (*m/z* 471); **10** – putative betulinic acid (*m/z* 455); **11** – ursolic & oleanolic acid (*m/z* 455).

Fig. 3. UHPLC MS / MS chromatograms for methanol – water extract of (**a**) callus, (**b**) leaves from shoot cultures, (**c**) leaves from field-grown plant and (**d**) fruits of *Chaenomeles japonica* (numerical values over peaks: mass of molecular ion).

TABLE 8. The content [mg g^{-1} d.w.] of selected pentacyclic triterpenoids and polyphenols in methanol-water extracts of *Chaenomeles japonica* biomass.

	PENTACYCLIC TRITERPENOIDS (PT) [mg g^{-1}] d.w.				POLYPHENOLS [mg g^{-1}] d.w.			
	Ursolic acid	**Oleanolic acid**	**Betulinic acid ***	**SUM OF PT**	**Chlorogenic acid**	**Dicaffeoylquinic acid***	**Epicatechin****	**Quercetin hexoside**
Callus	1.22 ± 0.09	1.26 ± 0.09	0.22 ± 0.01	2.70 ± 0.17	2.68 ± 0.15	–	0.08 ± 0.02	0.06 ± 0.01
Leaves from shoot culture	3.50 ± 0.31	1.16 ± 0.09	0.35 ± 0.03	5.01 ± 0.42	9.16 ± 0.40	7.33 ± 0.23	0.23 ± 0.02	0.31 ± 0.02
Leaves from field-grown plant	3.68 ± 0.26	1.36 ± 0.08	0.32 ± 0.02	5.36 ± 0.33	40.17 ± 2.30	0.51 ± 0.03	–	3.41 ± 0.18
Fruits	1.85 ± 0.19	1.51 ± 0.17	0.58 ± 0.05	3.94 ± 0.43	0.96 ± 0.05	–	0.30 ± 0.06	–

* expressed as oleanolic acid equivalent, **expressed as chlorogenic acid equivalent

(Moghaddam et al., 2012; Kikowska et al., 2016), it can be assumed that the effects of *C. japonica* extracts are probably due to the presence of pentacyclic triterpenes (ursolic acid, oleanolic acid, and betulinic acid) and phenolic acids (chlorogenic acid and its isomers) that occur both in the plant *in vivo* and in the raw materials from *in vitro* cultures, including calli.

Among various pentacyclic triterpenes, betulinic acid showed significant activity against *Staphylococcus aureus*. Even a better effect was observed for the combined treatment, i.e., betulinic acid with methicillin or vancomycin (Chung et al., 2011). On the other hand, betulinic acid isolated from *Vitex negundo* showed no activity against *Escherichia coli* (Chandramu et al., 2003). Oleanolic and ursolic acids isolated from *Alstonia scholaris* showed antibacterial activity against Gram-positive bacteria. Moreover, ursolic acid with ampicillin and tetracycline had a synergistic effect against *S. aureus* (Wang et al., 2016). The mechanism of action of triterpenoids has not been understood yet, but it has been postulated that they may exhibit antimicrobial activity by blocking cell division by inhibiting DNA synthesis (De Leon et al., 2005; Moghaddam et al., 2012).

It can be assumed that potential cosmetic preparations containing the extract from callus of Japanese quince culture, exhibiting the aforementioned properties, may inhibit development of *Acanthamoeba* trophozoites.

AUTHORS' CONTRIBUTION

MK: prepared and revised manuscript, analyzed and interpreted data, collected articles; MD and EH: conducted experiments, revised manuscript; BT: concept of investigations, provided plant material, revised manuscript; AW: conducted and analyzed biotechnological experiments, collected articles: JD: conducted and analyzed microbiological experiments, revised manuscript; AS and JŻ: conducted and analyzed phytochemical investigations, revised manuscript. The Authors declare that there are no conflicts of interest.

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