

EVALUATION OF ANTIAMOEBCIC AND ANTIMICROBIAL ACTIVITIES IN VITRO OF *CHAENOMELES JAPONICA* (THUNB.) LINDL. EX SPACH EXTRACTS

MAŁGORZATA KIKOWSKA^{1*}, MONIKA DERDA², BARBARA THIEM¹, AGATA
WŁODARCZYK¹, JOLANTA DŁUGASZEWSKA³, ANNA STOCHMAL⁴, JERZY ŻUCHOWSKI⁴
AND EDWARD HADAŚ²

¹Department of Pharmaceutical Botany and Plant Biotechnology,
Poznan University of Medical Sciences,
Św. Marii Magdaleny 14, 61-861 Poznań, Poland

²Department of Biology and Medical Parasitology,
Poznan University of Medical Sciences, Fredry 10, 61-701 Poznań, Poland

³Department of Genetics and Pharmaceutical Microbiology,
Poznan University of Medical Sciences, Święcickiego 4, 60-781 Poznań, Poland

⁴Department of Biochemistry and Crop Quality,
Institute of Soil Science and Plant Cultivation, State Research Institute,
Czartoryskich 10, 24-100 Puławy, Poland

Received July 19, 2019; revision accepted November 5, 2019

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 anti-amoebic drugs. Gram-positive bacteria *Staphylococcus aureus*, Gram-negative bacteria *Escherichia coli*, and a 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 anti-amoebicidal 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 anti-amoebicidal 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*

* Corresponding author, email: kikowska@ump.edu.pl

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-naphthaleneacetic 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).

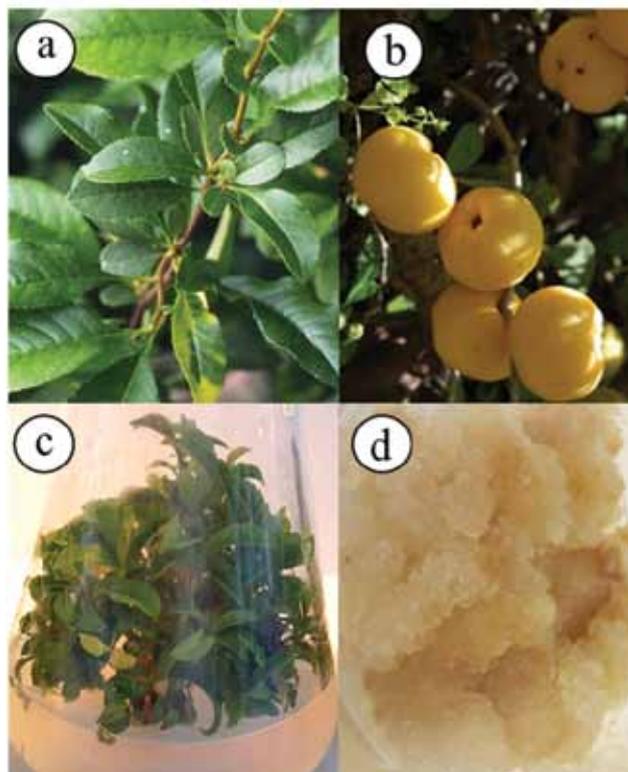


Fig. 1. *Chaenomeles japonica* (a) leaves from field-grown plant, (b) fruits, (c) leaves from shoot culture on MS media with 1.0 mg L⁻¹ BAP and 1.0 mg L⁻¹ IAA, (d) callus on MS with 2.0 mg L⁻¹ 2,4D + 0.2 mg L⁻¹ NAA.

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 antiamebic 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 trophozoite 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 amoebae 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 amoebae 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 trophozoite 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 IC₅₀ index was calculated for leaves from shoot culture extract (Table 5). On the second and third days of treatment, the IC₅₀ value was 0.30 mg mL⁻¹. The leaf extract from the field-grown plant also showed strong antiameobicidal activity, however, 3–4 times weaker than leaves from shoot culture. For this extract, the IC₅₀ 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 IC₅₀ was 2.30 mg mL⁻¹ and on the third IC₅₀ was 2.75 mg mL⁻¹. The extract from fruits exhibited the weakest effect among the tested extracts as on the second day the IC₅₀ 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					
	1 st		2 nd		3 rd	
	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					
	1 st		2 nd		3 rd	
	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					
	1 st		2 nd		3 rd	
	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					
	1 st		2 nd		3 rd	
	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 dicaffeoylquinic 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

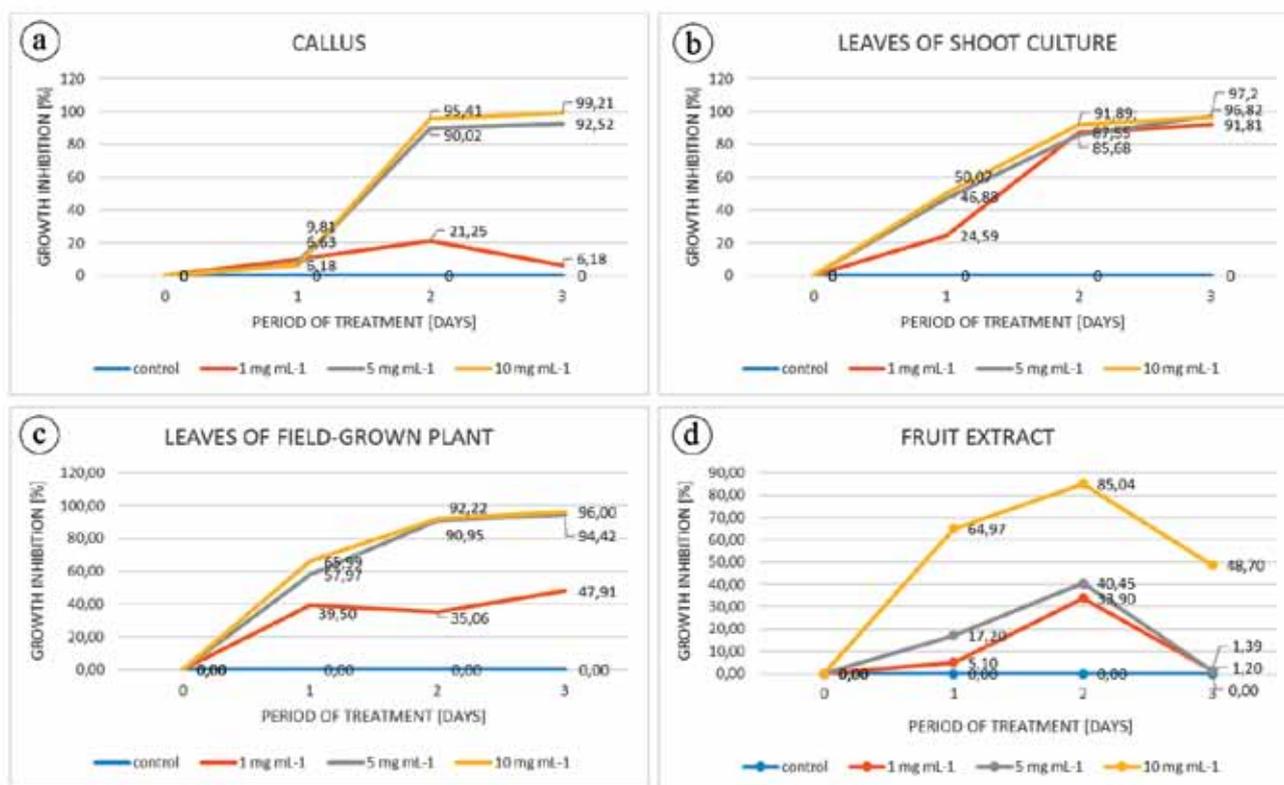


Fig. 2. Effect of *Chaenomeles japonica* (a) callus, (b) leaves from shoot culture, (c) leaves from field-grown plant and (d) fruit extracts [1 mg mL⁻¹, 5 mg mL⁻¹, 10 mg mL⁻¹] on inhibition of *Acanthamoeba trophozoites* proliferation in culture medium.

TABLE 5. Determination of IC₅₀ [mg mL⁻¹] for the studied extracts of *Chaenomeles japonica*.

Plant material	IC ₅₀ 1 st day	IC ₅₀ 2 nd day	IC ₅₀ 3 rd 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	–

IC₅₀ – 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; Niyati 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; Niyati 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		
	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd	1 st	2 nd	3 rd
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	87.5	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
<i>S. aureus</i>	20.0	160.0	5.0	40.0	2.5	10.0	10.0	40.0
<i>E. coli</i>	-	160.0	40.0	80.0	40.0	40.0	40.0	80.0
<i>C. albicans</i>	-	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; Niyayati et al., 2016) and phenolic acids

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 JŽ: conducted and analyzed phytochemical investigations, revised manuscript. The Authors declare that there are no conflicts of interest.

REFERENCES

- ALMUSAYEIB NM, MOTHANA RA, EL GAMAL AA, AL-MASSARANI SM, and MAES L. 2013. *In vitro* antiprotozoal activity of triterpenoid constituents of *Kleinia odora* growing in Saudi Arabia. *Molecules* 18: 9207–9218.
- BABALOLA I, and SHODE FO. 2013. Ubiquitous ursolic acid: a potential pentacyclic triterpene natural product – a review. *Journal of Pharmacognosy and Phytochemistry* 2: 214–222.
- CHANDRAMU C, MANOHAR RD, KRUPADANAM DG, and DASHAVANTHA RV. 2003. Isolation, characterization and biological activity of betulinic acid and ursolic acid from *Vitex negundo* L. *Phytoteraphy Research* 17: 129–134.
- CHEESMAN MJ, ILANKO A, BLONK B, and COCK IE. 2017. Developing new antimicrobial therapies: Are synergistic combinations of plant extracts/compounds with conventional antibiotics the solution? *Pharmacognosy Reviews* 11: 57–72.
- CHU DM, MILES H, TONEY D, NGYUEN C, and MARCIANO-CABRAL F. 1998. Amebicidal activity of plant extracts from Southeast Asia on *Acanthamoeba* spp. *Parasitology Research* 84: 746–752.
- CHUNG PY, NAVARATNAM P and CHUNG LY. 2011. Synergistic antimicrobial activity between pentacyclic triterpenoids and antibiotics against *Staphylococcus aureus* strains. *Annals of Clinical Microbiology and Antimicrobials* 10: 25.
- CUNHA WR, CREVELIN EJ, ARANTES GM, CROTTI AE, ANDRADE E SILVA ML, FURTADO NA, ALBUQUERQUE S, and FERREIRA DS. 2006. A study of the trypanocidal activity of triterpene acids isolated from *Miconia* species. *Phytotherapy Research* 20: 474–478.

- DE LEON L, BELTRAN B, and MOUJIR L. 2005. Antimicrobial activity of 6-oxophenolic triterpenoids. Mode of action against *Bacillus subtilis*. *Planta Medica* 71: 313–319.
- DE PABLOS LM, GONZALEZ G, RODRIGUES R, GARCIA GA, PARRA A, and OSUNA A. 2010. Action of a pentacyclic triterpenoid, maslinic acid, against *Toxoplasma gondii*. *Journal of Natural Products* 73: 831–834.
- DERDA M., and HADAŚ, E., 2015. The use of phytotherapy in diseases caused by parasitic protozoa. *Acta Parasitologica* 60(1): 1–8.
- DERDA M, HADAŚ E, CHOLEWIŃSKI M, SKRZYPCZAK Ł, GRZONDZIEL A, and WOJTKOWIAK-GIERA A. 2016. *Artemisia annua* L. as a plant with potential use in the treatment of acanthamoebiasis. *Parasitology Research* 115: 1635–1639.
- DERDA M, HADAŚ E, and THIEM B. 2009. Plant extracts as natural amoebicidal agents. *Parasitology Research* 104: 705–708.
- DERDA M, THIEM B, BUDZIANOWSKI J, WOJT WJ, and WOJTKOWIAK-GIERA A. 2013. The evaluation of the amoebicidal activity of *Eryngium planum* extracts. *Acta Poloniae Pharmaceutica* 70: 1027–1034.
- DU H, WU J, and LI H. 2013. Polyphenols and triterpenes from *Chaenomeles* fruits: Chemical analysis and antioxidant activities assessment. *Food Chemistry* 141: 4260–4268.
- EL-ZAWAHRY YA, REDA FM, and AZAZY WM. 2013. Synergistic effect of combination treatment by certain plant extracts and some antibiotics on the resistance of pathogenic bacteria to some common antibiotics. *Life Science Journal* 10: 3477–3489.
- FRATCZAK A, BYLKA W, and STUZIŃSKA-SROKA E. 2015. Fenolokwasy – budowa, działanie biologiczne i znaczenie w kosmetologii. *Polish Journal of Cosmetology* 18: 270–274.
- GNOATTO SC, VECHIA LD, LENCINA CL, DASSONVILLE-KLIMPT A, DA NASCIMENTO S, MOSSALAYI D, GUILLON J, GOSMANN G, and SONNET P. 2018. Synthesis and preliminary evaluation of new ursolic and oleanolic acids derivatives as antileishmanial agents. *Journal of Enzyme Inhibition and Medicinal Chemistry* 23: 604–610.
- HADAŚ E, DERDA M, NAWROT J, NOWAK G, and THIEM B. 2017a. Evaluation of the amoebicidal activities of *Centaurea bella*, *Centaurea daghestanica*, *Rhaponticum pulchrum* and *Tanacetum vulgare* against pathogenic *Acanthamoeba* spp. *Acta Poloniae Pharmaceutica* 74: 1827–1832.
- HADAŚ E, OZAROWSKI M, DERDA M, THIEM B, CHOLEWIŃSKI M, SKRZYPCZAK Ł, GRYSZCZYŃSKA A, and PIASECKA A. 2017b. The use of extracts from *Passiflora* spp. in helping the treatment of acanthamoebiasis. *Acta Poloniae Pharmaceutica* 74: 921–928.
- KASPRZAK W, and MAZUR T. 1972. Free living amoebae isolated from waters frequented by people in the vicinity of Poznań, Poland. Experimental studies in mice on the pathogenicity of the isolates. *Zeitschrift für Tropenmedizin und Parasitologie* 23: 391–398.
- KAYSER O, and KIDERLEN AF, CROFT SL. 2003. Natural products as antiparasitic drugs. *Parasitology Research* 90: 55–62.
- KIKOWSKA M, DŁUGASZEWSKA J, KUBICKA MM, KEDZIORA J, BUDZIANOWSKI J, and THIEM B. 2016. *In vitro* antimicrobial activity of extracts and their fractions from three *Eryngium* species L. *Herba Polonica* 62: 67–77.
- KIKOWSKA M, THIEM B, SLIWINSKA E, REWERS M, KOWALCZYK M, STOCHMAL A, and DŁUGASZEWSKA J. 2016. Micropropagation of *Eryngium campestre* L. via shoot culture provides valuable uniform plant material with enhanced content of phenolic acids and antimicrobial activity. *Acta Biologica Cracoviensia Series Botanica* 58: 43–56.
- KIKOWSKA MA, CHMIELEWSKA M, WŁODARCZYK A, STUZIŃSKA-SROKA E, ŻUCHOWSKI J, STOCHMAL A, KOTWICKA M, and THIEM B. 2018. Effect of pentacyclic triterpenoids-rich callus extract of *Chaenomeles japonica* (Thunb.) Lindl., ex Spach on viability, morphology and proliferation on normal skin fibroblasts. *Molecules* 23: 3009.
- KIKOWSKA M, WŁODARCZYK A, REWERS M, SLIWINSKA E, STUZIŃSKA-SROKA E, WITKOWSKA BANASZCZAK E, STOCHMAL A, ŻUCHOWSKI J, DŁUGASZEWSKA J, and THIEM B. 2019. Micropropagation of *Chaenomeles japonica*: a step towards production of polyphenol-rich extracts showing antioxidant and antimicrobial activities. *Molecules* 24: 1314.
- KUZMA Ł, DERDA M, HADAŚ E, and WYSOKIŃSKA H. 2015. Abietane diterpenoids from *Salvia sclarea* transformed roots as growth inhibitors of pathogenic *Acanthamoeba* spp. *Parasitology Research* 114: 323–327.
- MOGHADDAM MG, AHMAD FBH, and SAMZADEK-KERMANI A. 2012. Biological activity of betulinic acid: a review. *Pharmacology and Pharmacy* 3: 119–123.
- NABEED M, HEJAZI V, ABBAS M, KAMBOH AA, KHAN GJ, SHUMAZAID M, AHMAD F, BABAZADEH D, FANGFANG X, MODARRESI-GHAZANI, F, WENHUA L, and XIAOHUI Z. 2018. Chlorogenic acid: a pharmacological review and call for further research. *Biomedicine and Pharmacotherapy* 97: 67–74.
- NAHORSKA A, DZWONIARSKA M, and THIEM B. 2014. Fruits of Japanese quince (*Chaenomeles japonica* (Thunb.) Lindl. Ex Spach) as a source of bioactive compounds. *Postępy Fitoterapii* 15: 239–246.
- NIYATI M, DODANGEH S, and LORENZO-MORALES J. 2016. A review of the current research trends in the application of medicinal plants as a source for novel therapeutic agents against *Acanthamoeba* infections. *Iranian Journal of Pharmaceutical Research* 15: 893–900.
- PADZIK M, HENDIGER EB, SZAFLIK JP, and CHOMICZ L. 2017. Amoebae of the genus *Acanthamoeba* – pathological agents in humans. *Postępy Mikrobiologii* 56: 429–439.
- PARADES J, VOGLER O, ALEMANY R, GOMEZ-FLORIT M, FUNARI SS, RUIZ-GUTIERREZ V, and BARCELO F. 2011. Plant pentacyclic triterpenic acids as modulators of lipid membrane physical properties. *Biochimica et Biophysica Acta* 1808: 752–760.
- RASOULI H, FARZAEI MH, and KHODARAHMI R. 2017. Polyphenols and their benefits: A review. *International Journal of Food Properties* 20: 1700–1741.
- SIFAOURI I, LOPEZ-ARENCEBIA A, MARTIN-NAVARRO CM, REYES-BATLLE M, WAGNER C, CHIBOUB O, MEJRI M, VALLADARES B, ABOLERRABBA M, PINERO JE, and LORENZO-MORALES J. 2017. Programmed cell death in *Acanthamoeba castellanii* Neff induced by several molecules present in olive leaf extracts. *PLoS ONE* 12: e0183795.
- SIFAOURI I, LOPEZ-ARENCEBIA A, TICONA JC, MARTIN-NAVARRO CM, REYES-BATLLE M, MEJRI M, LORENZO-MORALES J, JIMENEZ AL, VALLADARES B, LOPEZ-BAZZOCCHI J, ABDERABBA M, and PINERO JE. 2014. Bioassay guided isolation and

- identification of anti-*Acanthamoeba* compounds from Tunisian olive leaf extracts. *Experimental Parasitology* 145: 111–114.
- SOKOŁOWSKA-WOŹNIAK A, SZEWCZYK K, and NOWAK R. 2002. Phenolic acids from *Cydonia japonica* Pers. *Herba Polonica* 48(8): 214–8.
- SIMELANE MB, SHONHAI A, SHODE FO, SMITH P, SINGH M, OPOKU AR. 2014. Anti-plasmodial activity of some Zulu medicinal plants and of some triterpenes isolated from them. *Molecules* 18: 12313–12323.
- STEELE JC, WARHURST DC, KIRBY GC, and SIMMONDS MS. 1999. *In vitro* and *in vivo* evaluation of betulinic acid as an antimalarial. *Phytotherapy Research* 13: 115–119.
- TAN N, KALOGA M, RADTKE OA, KIDERLEN AF, OKSUZ S, ULUBELEN A, and KOŁODZIEJ H. 2002. Abietane diterpenoids and triterpenoic acids from *Salvia cilicica* and their antileishmanial activities. *Phytochemistry* 61: 881–884.
- TANVEER T, BIN-DUKHYL AA, NAWAZ S, and MATIN A. 2018. Therapeutic potential of folk plants used against pathogenic and opportunistic free-living amoeba (*Acanthamoeba*, *Balamuthia* and *Naegleria*); A review. *International Journal of Cell Science and Molecular Biology* ID.555628.
- VAN BAREN C, ANAO I, LEO D, LIRA P, DEBENEDETTI S, HOUGHTON P, CROFT S, and MARTINO V. 2006. Triterpenic acids and flavonoids from *Satureja parvifolia*. Evaluation of their antiprotozoal activity. *Journal of Bioscience* 61: 189–192.
- VASHIST H, and JINDAL A. 2012. Antimicrobial activities of medicinal plants – review. *International Journal of Research in Pharmaceutical and Biomedical Sciences* 3: 222–230.
- WANG CM, CHEN HT, WU ZY, JHAN YL, SHYU CH, and CHOU CH. 2016. Antibacterial and synergistic activity of pentacyclic triterpenoids isolated from *Alstonia scholaris*. *Molecules* 1: 139.
- XU YN, KIM JS, KANG SS, SON KH, KIM HP, CHANG HW, and BAE K. 2002. A new acylated triterpene from the roots of *Chaenomeles japonica*. *Chemical and Pharmaceutical Bulletin* 50: 1124–1125.
- YOGEEWARI P, and SRIRAM D. 2005. Betulinic acid and derivatives: a review on their biological properties. *Current Medicinal Chemistry* 12: 657–666.